Development of a Diagnostic-Serological Assay for Ovine Herpesvirus-2

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A thesis submitted for the degree of Doctor of Philosophy in the College of Medicine and Veterinary Medicine, University of Edinburgh

Declaration

The work presented in this thesis was carried out at the Moredun Research Institute, Edinburgh. The experimental work and interpretation of the results were undertaken by the author. Contributions to the work in this thesis by colleagues are fully acknowledged in the text.

This work has not been nor is currently being submitted for candidature for any other degree.

Sarah Fraser
April 2007

Acknowledgements

I would like to thank the following people for their help during the course of my study and in the preparation of this thesis:

My supervisors Dr. George Russell and Dr. Bernadette Dutia for their constant encouragement, support and constructive advice throughout my time at Moredun.

Dr. Peter Nettleton for all his help and advice, particularly in the establishment and validation of the ELISA test.

Dr. David Deane for all his help and advice with the FPLC, gel filtration and purification methods and all his useful discussions.

I would also like to thank Miss Iris Campbell for all her help and advice regarding cell culture, preparation of ConA blast cells, inoculation of rabbits for MCF antiserum as well as all the information regarding the virus neutralisation assay performed in the vaccination trial. I would also like to thank Janice Gilray for the MCF serum and WC11 virus stock, Dawn Grant for performing the OvHV-2 Real-time PCR and nested PCR, and Mr. Kevin M^{c}Lean for performing the mass spectrometry.

Moredun Research Institute and Moredun Scientific Limited for funding this project.

My particular thanks to Linda Marriott, Ann Percival, Thonur Leenadevi, Joanne Crawford and Inga Dry for their continued support and encouragement in and out of the lab.

Lastly I would like to thank my family and my friends for their constant support and encouragement throughout this study.

Abstract

Malignant catarrhal fever (MCF) is a sporadic but fatal lymphoproliferative viral disease of cattle, deer and other ruminants. The causative agents are highly-cell-associated herpesviruses of the subfamily *gammaherpesvirinae* including Alcelaphine herpesvirus-1 (AlHV-1) and Ovine herpesvirus-2 (OvHV-2). The identification of MCF-affected animals is based on the detection of antibodies to AlHV-1 in a variety of assays including indirect-immunofluorescence tests (IFAT) and a competitive-inhibition enzyme-linked immunosorbent assay (CI-ELISA), although histopathological examination remains the most recognised diagnostic procedure. PCR assays are also available for the detection of MCFV DNA.

The aim of this project was to develop a serological assay based on recombinant antigens derived from the OvHV-2 genome and validation of the ELISA test using clinical serum samples. Eight OvHV-2 genes were selected for expression in three bacterial systems. Four genes could be expressed in at least one system, but only a fragment of the Ov8 protein and ORF65 were found to be recognised by anti-MCF sera. Additionally Ov2.5, expressed in mammalian cells, was not recognised by anti-MCF sera. Three genes were expressed in an *in vitro* transcription-translation system (Ov8, Ov8.5 and ORF65). Protein purification, using epitope-tag affinity purification and conventional chromatography, was unsuccessful so that these polypeptides could not be evaluated as recombinant ELISA antigens, requiring further work on large scale expression and purification.

Due to the lack of recombinant antigen expression, crude antigen preparations from cultured AlHV-1 and OvHV-2 infected cells were analysed. Antigens suitable for the detection of MCF virus specific serum antibodies could not be extracted from an OvHV-2 infected cell line but AlHV-1 WC11 antigen could be used to differentiate MCF-positive and -negative sera. An ELISA based on this crude antigen was established, designated WC11-ELISA. Initial validation revealed over 93% concordance between WC11-ELISA and commercial ELISA, while a comparison with serological assays, a PCR test and final veterinary evaluations, showed good agreement between tests. The WC11-ELISA showed 57% agreement to PCR while commercial CI-ELISA showed 66% agreement for the same samples. This indicates the value of the ELISA as an economical, rapid and specific serological assay for the detection of antibodies against MCFV.

Potentially antigenic components of the ELISA lysate were analysed by western blotting in comparison with other bovine herpesviruses, and fractionation by gel filtration showed clear antigenic bands in the eluent. Proteomic analysis of antigenic bands identified proteins encoding viral thymidine kinase (ORF21), a virus capsid protein (ORF5), ORF52 (homologue to tegument protein HSV UL49) and a viral antigen (ORF54) which encodes a dUTPase.

The simplicity of the WC11-ELISA and its lack of cross-reactivity make this assay a reliable, accessible and low cost tool for routine MCFV diagnosis and epidemiological studies.

CONTENTS

Chapter Two: General Materials and Methods

LIST OF ABBREVIATIONS USED IN TEXT

Ag	- Antigen
AHV-1	- Alcelaphine Herpesvirus Type-1
AHV-2	- Alcelaphine Herpesvirus Type-2
AIDS	- Acquired immunodeficiency syndrome
AU	- Absorbance unit
BCA	- Bicinchoninic acid
BEK	- Bovine Embryonic Kidney
BL	- Burkitt's lymphoma
BoHV-1	- Bovine Herpesvirus Type-1 (infectious bovine rhinotracheitis)
BoHV-2	- Bovine herpesvirus-2
BoHV-4	- Bovine Herpesvirus-4
BJ1035	- OHV-2 virus strain originally derived from naturally infected bovine
BT	- Bovine turbinate
C500	- AHV-1 virus strain isolated from wildebeest derived MCF
°C	- Degrees Centigrade
CF	- Complement Fixation
CPE	- Cytopathic Effect
DNA	- Deoxyribonucleic acid
EBV	- Epstein Barr Virus
EHV-2	- Equine Herpesvirus Type-2
ELISA	- Enzyme-Linked Immunosorbent Assay
FBS	- Fetal bovine serum
FITC	- Fluorescein
FPLC	- Fast Protein Liquid Chromatography
g	- Grams/
GPCR	- G-protein-coupled receptor
HBSS	- Hanks Balanced Salts Solution
HCl	- Hydrocholiric acid
HHV-8	- Human Herpesvirus-8 (Kaposi's sarcoma virus).
HL	- Hodgkin's lymphoma
HRP	- Horse Radish Peroxidase
HSV	- Herpes Simplex Virus
HVS	- Herpesvirus saimiri
IBR	- infectious bovine rhinotracheitis (bovine herpesvirus type-1)
IgG	- Immunoglobulin G antibody
IgM	- Immunoglobulin M antibody
IFAT	- Immunofluorescence antibody test
IIFT	- Indirect Immunofluorescence test
IM	- Infectious mononucleosis
IMDM	- Iscoves medium
IVP	- Infectious pustular vulvovaginititis (BoHV-1)
Kb	- Kilobase
kDa	- KiloDalton
KS	- Kaposi's sarcoma (HHV-8)

KSHV	- Kaposi's sarcoma herpesvirus
LCV	- gammaherpesvirus genus: *Lymphocryptovirus* (γ_1-herpesvirus)
Ni	- Nickel
MAbs	- Monoclonal Antibodies
MCF	- Malignant catarrhal fever
MCFV	- Malignant catarrhal fever virus
MCD	- Multicentric Castleman's disease
mM	- Millimolar
mwt	- Molecular Weight
NPC	- Nasopharyngeal carcinoma
ng	- nanograms
nm	- Nanometer
NP-40	- Nonylphenylpolyethylene glycol (Nonident-P40)
NTA	- nitriloacetic acid (Ni-NTA purification)
O.D	- Optical Density
ORFs	- Open Reading Frames
OvHV-2	- Ovine Herpesvirus-2
PBS	- Phosphate buffered saline
PBST	- PBS plus 0.05% Tween-20
PBSTH	- PBST containing 10% horse serum
PCR	- Polymerase Chain Reaction
PEL	- Primary effusion lymphoma
RDV	- Gammaherpesvirus genus: *Rhadinovirus* (γ_2-herpesviruses)
rpm	- per minute
SA MCF	- Sheep Associated Malignant Catarrhal Fever.
SDS	- Sodium Dodecyl Sulphate
STP-C	- Saimiri transformational protein strain C
TAE	- Tris-Acetate-EDTA
TBE	- Tris-Borate-EDTA
TBS	- Tris-Buffered Saline
TMB	- Tetramethylbenzidine Peroxidase
µl	- Microlitre
µM	- Micromolar
VN	- Virus Neutralisation test
VZV	- Varicella zoster virus
WA-MCF	- Wildebeest-derived MCF
WC11	- AHV-1 virus strain isolated originally from wildebeest calf.
X-GAL	- 5-bromo-4-chloro-3-indolyl-bD-galactoside

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Chapter One
General Introduction

1.1. Introduction

Malignant catarrhal fever (MCF) is an acute, sporadic and usually fatal disease affecting domestic and wild ruminants and in particular species of the subfamily Bovinae and family Cervidae. MCF is characterised by the recognition of lymphoid cell accumulations in non-lymphoid organs and T-lymphocyte hyperplasia in lymphoid organs (Office des International, 2004), and can be caused by either of two gammaherpesviruses; Alcelaphine herpesvirus-1 (AlHV-1) and Ovine herpesvirus-2 (OvHV-2). The natural hosts of AlHV-1 are wildebeest, while the natural hosts of OvHV-2 include all species of wild and domestic sheep. Affected carriers remain asymptomatic. AlHV-1 is found in cattle in regions of Africa and in a variety of ruminant species in zoological collections world-wide, while OvHV-2 is a welfare issue of economic significance worldwide.

The disease may present a variety of clinical manifestations ranging from the acute form were death may occur within hours, to chronic cases characterised by high fever, bilateral corneal opacity, profuse catarrhal discharges from the eye and nose and necrosis of the muzzle. Current diagnosis is reliant on histopathological examination, though detection of viral DNA by PCR and serological assays are available.

1.2. The Herpesviruses

Herpesviruses are a large, clearly defined group of viruses (Bowman *et al.*, 2003) found in all species of animal and birds. Several herpesviruses have been isolated from humans, including; herpes simplex virus 1 and 2 (HSV-1, HSV-2), human cytomegalovirus (HCMV), varicella-zoster (VZV), Epstein Barr virus (EBV) and human herpesvirus 6 and 7 (HHV-6 & 7) and Kaposi's sarcoma associated herpesvirus (KSHV, HHV-8). Of over 130 herpesviruses identified, approximately 100 have been partially characterised to date (Roizman, 2001; Minson, 2000), and the genomes of at least 19 have been sequenced. Within livestock species these include equine herpesviruses 1, 2 and 4, bovine herpesvirus-1, bovine herpesvirus-5, alcelaphine herpesvirus-1 and ovine herpesvirus-2.

Herpesviruses are fragile and are therefore do not survive well outside the body. In nature, each herpesvirus is closely associated with a single host species,

where transmission of these viruses requires close contact, and in particular mucosal contact. Subsequently closely confined populations allow the possibility for modes of transmission such as via sneezing and coughing. The virus is released in aerosol droplets were it might be inhaled by a susceptible host (Roizman and Pellett, 2001). Herpesviruses survive in nature due to persistent, latent infections from which the virus is intermittently reactivated and shed.

1.2.1. Biological Properties of Herpesviruses

All known herpesviruses share four significant biological properties (Roizman, 2001).

1. All herpesviruses specify a large range of enzymes involved in nucleic acid metabolism, DNA synthesis and possibly in the processing of proteins.
2. The synthesis of viral DNAs and assembly of capsids occurs within the nucleus.
3. Production of infectious progeny virus is invariably accompanied by the irreversible destruction of the infected cell
4. They possess the ability to remain latent in their natural hosts. If the virus is latent, the viral genome takes the form of closed, circular molecules.

Originally, herpesviruses were categorised on the basis of their biological properties. However, the primary means for identification is through virus structure. With the determination of the DNA sequence of the viral genome, the herpesviruses have now been classified into genera, based on DNA sequence homology and the antigenic relatedness of the viral proteins.

Some herpesviruses have a wide host range, and can multiply and infect cells rapidly, such as HSV-1. Others possess a narrow host range, such as EBV, or have long replicating life cycles, such as HCMV. Although all herpesviruses have a latent phase, differences arise in the exact site of latency.

1.2.2. Virion properties and structure

The structure of the herpes virion is complex with characteristic symmetrical and nonsymmetrical components. Herpesvirus virions are enveloped and are about 200-250nm in diameter (Figure 1.1). A typical virion consists of **(a)** a core containing linear double stranded DNA, **(b)** an icosahedral nucleocapsid, **(c)** the tegument and **(d)** a typical lipoprotein membrane designated the envelope (Castro *et al.*, 1985; Van Regenmortel *et al.*, 2000; Flach *et al.*, 2002; Bowman *et al.*, 2003).

The core is comprised of the viral genome (125-245kbp), which is packaged as a single, linear, double-stranded DNA molecule (Davidson, 2002). This is enclosed in a protein capsid. The structural features of this protein capsid are characteristic of herpesviruses (100nm in diameter and 162 capsomeres) (Roizman, 1996). Surrounding the capsid is a globular layer, known as the tegument, a term introduced by Roizman and Furlong (1974), which is employed to describe the structures present between the capsid and the envelope. These structures have no distinctive feature, though contain many proteins and polypeptides, some of which are not required *in vitro* and are therefore not needed for the formation of virions. These proteins and polypeptides vary among the different members of the *Herpesviridae* family (Bowman *et al.*, 2003).

On the outer surface of the virus structure is the lipoprotein envelope, a lipid bilayer that is associated to the outer surface of the tegument, and is known to contain at least 10 integral membrane proteins, including a variety of viral glycoproteins required for entry into the host cell (Van Regenmortel *et al.*, 2000).

1.2.3. Classification of herpesviruses

Based upon the general molecular characterisations, such as nucleotide sequence and phylogenetic analyses, the family *Herpesviridae* is divided into three subfamilies. They are termed; *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* (Roizman, 1996). Viruses belonging to the subfamilies of herpesviruses are summarised in Table 1.1.

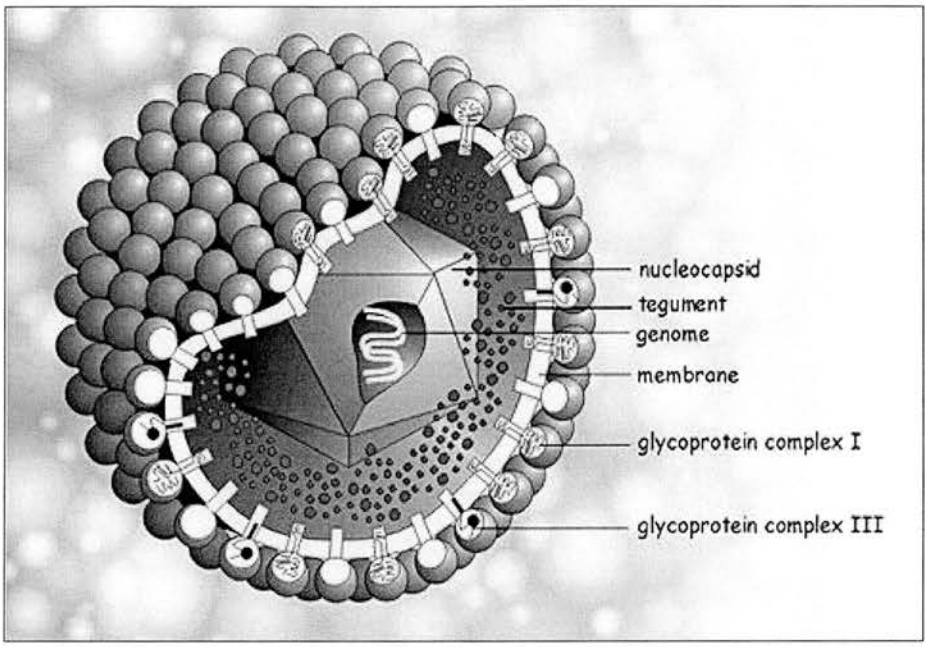


Figure 1.1: Diagram of herpesvirus. Main structural components of the herpesvirus virion. Herpesvirus virions are approximately 200-250nm in diameter. A typical virion consists of **(a)** a core containing linear double stranded DNA, **(b)** an icosahedral nucleocapsid, **(c)** the tegument and **(d)** a typical lipoprotein membrane designated the envelope. **Illustration taken from http://www.spectrosciences.com.**

1.2.3.1. Alphaherpesvirinae

Members of this group are classified on the basis of a relatively short reproductive cycle, efficient destruction of infected cells and a variable host range. Alphaherpesviruses commonly possess the capacity to establish latent infections in neurons. The subfamily includes the genera *Simplexvirus* (e.g. Herpes simplex-1 and 2, Bovine herpesvirus-2), *Varicellovirus* (e.g. Varicella zoster virus, Bovine herpesvirus-1) and *Mardivirus* and *Iltovirus* (Marek's disease). Commonly, members of the genera *Simplexvirus* are viruses found in primates while members of the genera *Varicellovirus* are found in a wide range of mammalian hosts (Van Regenmortel *et al.*, 2000). *Mardivirus* and *Iltovirus* viruses are found in birds and are commonly associated with malignancy such as shown in Marek's disease.

Herpes simplex virus

Herpes simplex virus was the first human herpesvirus to be identified. In 1893, human transmission of the virus was described from one individual to another (Whitley, reviewed in Fields Virology). Host immune responses were described in the 1930's, defining the presence of neutralising antibodies in serum of infected patients. The recurrence of lesions after the primary infection resulted in the demonstration of a unique biological property whereby viruses can recur in the presence of humoral immunity of the host. This response was known as the reactivation of latent infection (Whitley, reviewed in Fields Virology).

Advances in molecular technology have resulted in an increased understanding of epidemiology and infection and led to the discrimination of HSV types 1 and 2. HSV-1 (human herpesvirus 1, HHV-1) and HSV-2 (human herpesvirus 2, HHV-2) are widespread human pathogens, generally associated with oral and genital infections. Nahmias and Dawdle (1968) showed antigenic and biological variations between these two types. HSV-1 is frequently associated with non-genital infections causing ocular herpes and is an important cause of encephalitis and infects 60-80% of people worldwide (Cunningham *et al.*, 2006). HSV-2 is commonly associated with genital disease, where prevalence between countries varies (7-80%).

Name	Host	Disease
Alphaherpesviruses		
Herpes simplex-1	Human	Cold sores, keratitis
Herpes simplex-2	Human	Genital herpes
Varicella zoster virus	Human	Chicken pox and shingles
Bovine herpesvirus-1	Bovine	Infectious bovine rhinotracheitis, Genital disease, abortions
Bovine herpesvirus-2	Bovine	Bovine herpes mammilitis, Pseudo-lumpy skin disease
Feline herpesvirus-1	Cats	Feline viral rhinotracheitis
Porcine herpesvirus-1	Pigs	Pseudorabies (Aujzesky's disease)
Equine herpesvirus-1	Horses	Abortion, neurological disease
Gallid herpesvirus-2	Chickens	Marek's disease
Betaherpesviruses		
Human cytomegalovirus	Human	Mononucleosis, multi-system disease in immunocompromised patients
Porcine cytomegalovirus	Pigs	Rhinitis in piglets
Murine cytomegalovirus	Mice	
Human herpesvirus-6	Human	Fever and rash in children
Human herpesvirus-7	Human	Mononucleosis in adults
Gammaherpesviruses		
Epstein-Barr virus	Human	Infectious mononucleosis, Burkitt's lymphoma, PTLD, nasopharyngeal carcinoma,
Kaposi's sarcoma associated herpesvirus	Human	Kaposi's sarcoma, Body cavity lymphomas, MCD
Equine herpesvirus-2	Horses	? Respiratory disease in foals
Bovine herpesvirus-4	Cattle	? Conjunctivitis, metritis, mastitis
Herpesvirus saimiri	Squirrel monkey	Fatal lymphoproliferations in cottontail rabbits & new world monkeys e.g. marmosets
Herpesvirus ateles	Spider monkey	Lymphomas in new world primates
Rhesus rhadinovirus	Rhesus monkey	Lymphoproliferative disease in immunosuppressed host
Murine gammaherpesvirus-68	Murid rodents	Lymphomas
Porcine lymphotropic herpesvirus-1	Pigs	
Alcelaphine herpesvirus-1	Wildebeest	Malignant catarrhal fever in cattle, deer and other ruminants
Ovine herpesvirus-2	Sheep	

Table 1.1: Examples of herpesviruses. Subgrouping as described in section 1.2.3 (Roizman, 2001).

Bovine Herpesvirus-1 (BHV-1)

Bovine herpesvirus-1 is a member of the herpesvirus subfamily *Alphaherpesvirinae*, genus *Varicellovirus*. BHV-1, commonly associated with infectious bovine rhinotrachetitis (IBR) and infectious pustular vulvovagnititis (IPV) was first described in Europe in 1913 (Babiuk *et al.*, 2004). The virus occurs worldwide, where aside from the bovine host, other ruminants such as goats, sheep (Whetstone and Evermann, 1988; Thiry *et al.*, 2006), deer (Thiry *et al.*, 2006) and buffalo (Ibrahim *et al.*, 1983) may be susceptible. In susceptible animals, the virus can cause mild to severe clinical disease.

Clinical symptoms include rhinotracheitis, conjunctivitis, reduced milk yield and genital infections (Hurk, 2006). Although it affects cattle of all ages it is commonly recognised in animals over six months old. This suggests young animals are protected as a result of passive immunity, possibly acquired from colostrum. Transmission of the virus is through direct contact with infected animals nasal secretions and excretions entering food and water sources. Diagnosis of BHV-1 is reliant on either direct identification of the agent (histopathology) or assays to detect virus or antibody in the host (Babiuk *et al.*, 2004). Viral antigens can be detected in nasal or genital swabs by assays including; immunofluorescence, ELISA, virus neutralisation and immunohistochemistry. These tests prove beneficial as a method of control, where herds can be screened for BHV-1 virus.

The BHV-1 genome consists of double-stranded linear DNA approximately 140kbp and codes for over 70 proteins (Mayfield *et al.*, 1983). Based on genomic characterisation, two subtypes of bovine herpesviruses have been recognised, designated subtype 1 (BHV-1.1) and subtype 2 (BoHV1.2) (Miller *et al.*, 1995; D'Arce *et al.*, 2002; Spilka *et al.*, 2005). The epidemiological importance of these two disease subtypes, however, remains unknown (Spilka *et al.*, 2005).

Bovine Herpesvirus-2 (BHV-2)

Bovine herpesvirus-2 is a member of the subfamily *Alphaherpesvirinae*, genus *Simplexvirus*. BHV-2 was first reported in South Africa in 1957 (Reviewed by Gibbs, 2004), and later in the UK in 1964 (Martin *et al.*, 1966) and is commonly associated to two clinical forms: bovine herpes mammilitis and pseudo-lumpy skin

disease (Imai *et al.*, 2004). BHV-2 infections are commonly subclinical, however some clinical signs can be presented including ulcerated or necrotic lesions of the udder and teat, oedematous teat swelling and inflamed dermis. In dairy cattle, a decline in milk production and mastitis may be observed resulting in economic losses (Imia *et al.*, 2002). Initial diagnosis is primarily based on the presence of the characteristic lesions on the udder and teats. Virus isolation and histopathology, including microscopic examination of skin biopsies, are the most recognised methods for the detection of the virus. However, serological assays such as virus neutralisation may also confirm the presence of antibodies against the virus (Imai *et al.*, 2004). PCR methods have also been used in the diagnosis of viral diseases including BHV-2 (Imai *et al.*, 2002). Pseudo-lumpy skin disease can be characterised by generalised skin lesions (Imai *et al.*, 2004).

The mode of BHV-2 transmission is still unclear, but it is assumed to be a mechanical transmission by insect vectors. Domestic cattle such as dairy herds are affected, the significance of BHV-2 in wildlife is unknown and raises further doubt regarding the route of transmission (Imai *et al.*, 2004).

1.2.3.2. Betaherpesvirinae

Members of the subfamily *betaherpesvirinae* have a restricted host range and are species-specific. Unlike *alphaherpesviruses*, members of this group have a long reproductive cycle and the infection progresses slowly in culture. The virus remains cell-associated and may be maintained in a latent form in monocytes, in regions such as the kidneys, secretory glands and other tissues. Members of this group include the genera *Cytomegalovirus* (e.g. Human cytomegalovirus, Porcine cytomegalovirus), *Muromegalovirus* (e.g. murine cytomegalovirus) and *Roseolovirus* (e.g. Human Herpesvirus-6 [HHV-6] and Human Herpesvirus-7 [HHV-7]).

1.2.3.3. Gammaherpesviridae

The subfamily *gammaherpesvirinae* is comprised of herpesviruses which are lymphotropic. Members of this subfamily have a narrow host range. The viruses establish latency in lymphocytes and lymphoid tissues and may cause lytic infections

in fibroblastic and epithelial cells. Infection is therefore associated with lymphoproliferative disorders, and malignancies of lymphoid tissues.

Gammaherpesviruses are divided into two groups; γ_1-herpesvirus (genus *Lymphocryptovirus*) and γ_2-herpesviruses (genus *Rhadinovirus*) (Honess & Watson, 1977; Roizman, 1982; Honess, 1984; Roizman *et al.*, 1992).The *Lymphocryptoviruses* (LCV) include viruses such as Epstein-Barr virus (EBV), known to infect B lymphocytes in culture, the site of latent infection. The *Rhadinoviruses* (RDV) include viruses such as human-herpesvirus-8 (Kaposi's sarcoma virus), Herpesvirus saimiri (HVS), equine herpesvirus-2 (EHV-2) and bovine herpesvirus-4 (BoHV-4) (Plowright *et al.*, 1972), alcelaphine herpesvirus-1 and ovine herpesvirus-2, where latent infection is reported in T and B lymphocytes (Van Regenmortel *et al.*, 2000).

1.2.4. Herpesvirus genome

The composition, size and organisation of the genome of herpesviruses are found to vary significantly. The majority of the DNA from virions is linear, double stranded molecules ranging in size from 125-235kb. Furthermore the percentage of guanine plus cytosine (G + C) is found to vary between 32 and 75% (Roizman, 1996). Although herpesviruses share some significant biological properties, they are also shown to be vastly different in others. While some have a wide cell range, and can multiply efficiently, rapidly destroying the cells they infect (HSV-1, HSV-2), others have a limited host range (such as EBV and HHV-6) and multiply slowly (human cytomegaloviruses (HCMV)).

According to DNA sequence arrangement, herpesviruses may be divided into six groups (Figure 1.2). These are termed A-F (Roizman, 2001).

Group A - A large sequence from one terminus is shown directly repeated at the other terminus. An example of this is channel catfish herpesvirus. Viruses of the subfamily *Betaherpeviridae Roseolovirus* are represented by this group (Human herpesviruses 6 & 7).

Group B - The terminal sequence is directly repeated frequently at both termini. Additionally, the number of reiterations at both termini may also vary. This is shown in the subfamily *Gammaherpesvirus rhadinovirus* viruses which include Herpesvirus saimiri, Alcelaphine herpesvirus-1 and Ovine herpesvirus-2.

Group C - In genomes such as Epstein-Barr virus (EBV), there are less terminal reiterations, though there may be other internal unrelated sequences (normally greater than 100bp) which are directly repeated and may subdivide unique sequences of the genome into several sections. This group represent the subfamily *Gammaherpesvirus lymphocryptovirus*.

Group D - In group D viral genomes, the subfamily *alphaherpesvirus Varicellovirus* such as Varicella zoster virus (VZV) and bovine herpesvirus-1 (BHV-1), the sequence at one terminus (TR) is repeated in an inverted orientation internally (IR). This divides the unique region into long unique (U_L) and short unique (U_S) regions. The short region can invert, resulting in two isomers of viral DNA.

Group E - One terminus contains N repeats of sequence A next to a longer sequence B. The other terminus has one directly repeated sequence A next to a sequence C. The terminal sequences are inserted in an inverted form internally (B' A'_N, C'). Both the long and short regions can invert, forming four isomers. This is observed in viruses of the subfamilies *alphaherpesvirus Simplexvirus* (Bovine herpesvirus-2, Herpes Simplex virus 1 & 2) and *Betaherpesvirus Cytomegalovirus* (Human cytomegalovirus).

Group F - The two termini are not identical in members of group F, and are not repeated directly. Group F are exemplified by the tupaia herpesvirus, Tupaiid herpesvirus-1, a virus of tree shrews. (Roizman & Pellet, 2001).

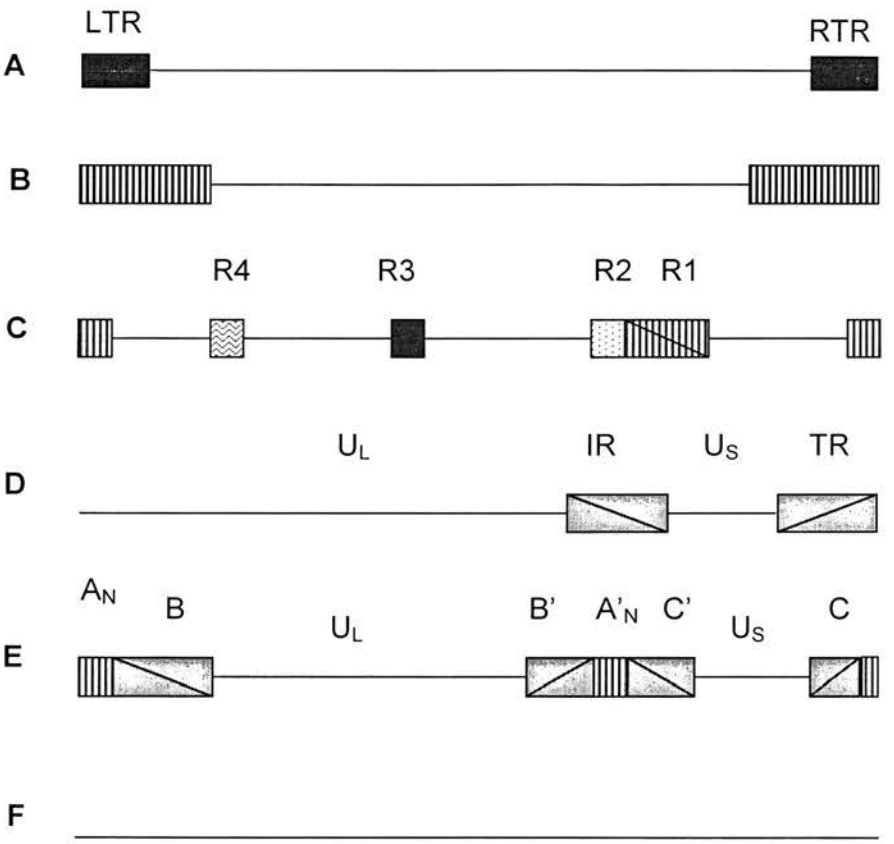


Figure 1.2: Sequence Arrangements of Herpesvirus Genomes. The sequence arrangements of the six categories of viral genomes of the herpesvirus family are shown (Illustration taken from Roizman, 2001).

1.2.5. Herpesvirus Life Cycle and Replication

The herpesviruses life cycle has two stages, the lytic stage and the latent stage. The lytic cycle is comprised of viral replication, production of new virions and the subsequent release from the cell, which normally causes the infected cell to lyse. The latent stage involves the virus genome persisting in the cell as an episome, with no production of new virions and expression of only a small subset of viral genes. As many replication genes are conserved within herpesviruses, the principles of replication are likely to be applicable to most herpesviruses and are known to involve numerous stages. As HSV-1 is well characterised, the general process of replication has been studied in detail (Roizman and Knipe, 2001) and is described here as a prototype of the process in other herpesviruses (Figure 1.3).

Virus attachment and entry

Initially, the virus attaches to cell surface receptors, which involves the interaction between viral glycoproteins present in the viral envelope. Glycoproteins (gPs) are known to mediate attachment and penetration to the cell surface. There are ten viral glycoproteins designated gB, gC, gD, gE, gG, gH, gI, gK, gL and gM, although not all subfamilies have all gPs and some have other gPs such as EBV gP340. The envelope glycoproteins, gB and gC, are essential for attachment, as they bind to cell surface glycosaminoglycans such as surface heparin sulphate or chondroitin sulfate. (Reviewed in Rajčáni & Vojvodvá, 1998). The subsequent step involves the interaction of the viral glycoproteins with several cellular co-receptors, which is necessary for fusion of the viral envelope with the host cell membrane. This involves the interaction of glycoprotein gD and cellular receptors. For HSV-1, these cellular receptors include members of the tumour necrosis factor (TNF) receptor family, members of the immunoglobulin superfamily, designated nectins on the basis of their cellular function, and the 3-O-sulphated heparin sulphates (reviewed in Spears *et al.*, 2000).

Fusion requires the interaction of gD, gB and the heterodimer gH-gL, although their precise function is unknown. Glycoproteins gI and gE form a viral Fc

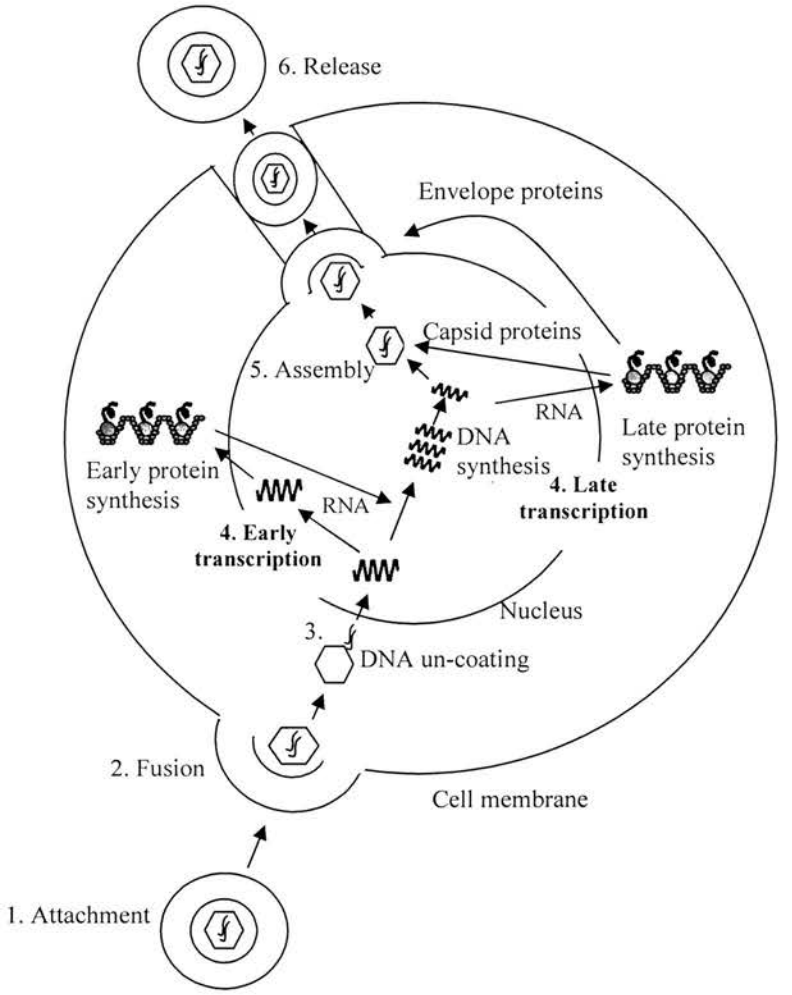


Figure 1.3: The replication cycle of Herpes Simplex virus. (1) Specific proteins in the viral envelope attach to host cell receptors on the cell membrane (2) viral envelope fuses with the cell membrane releasing the nucleocapsid directly into the cytoplasm. (3) the viral DNA is transported into the nucleus (4) the viral DNA is transcribed into early mRNAs which are transported to the cytoplasm for the translation of early proteins, which participate in the replication of the virus DNA. The viral DNA is then transcribed into the late mRNAs which exit to the cytoplasm for translation into the late (nucleocapsid and envelope) proteins (5). The capsid proteins assemble (6) The nucleocapsids are enveloped by budding through the nuclear membrane, and the mature viruses are released from the cell through cytoplasmic channels. Figure taken from (http://www.bact.wisc.edu/themicrobialworld/Viruses1.html

receptor and are present following penetration. Following fusion, some tegument proteins remain in the cytoplasm while others are transported to the nucleus or remain associated with the nucleus. The capsids, with associated tegument proteins, are then transported to the nuclear pore. At the nuclear pore, the nucleocapsid releases DNA into the nucleus. The tegument proteins are introduced to the cell with the capsid and may play a role in preparing the host cell for viral replication.

After entering the cell nucleus, the viral DNA circularises and viral gene expression initiates. Transcription and replication of viral DNA occur in the nucleus, while viral proteins are synthesised in the cytoplasm. The capsid is assembled in the nucleus. Viral DNA is transcribed by host RNA polymerase and mRNA is translated in the cytoplasm (Roizman & Spears, 1990). Gene expression in the lytic phase occurs in a chronological order that can be grouped into three categories; immediate early (IE), early (E) and late (L).

1.2.6. Viral replication

The cellular cytoskeleton is thought to mediate the transport of the capsid to the nucleus. Capsids of HSV-1 accumulate at nuclear pores and release viral DNA into the nucleoplasm. The capsid therefore may act as a vector for transporting viral DNA, such as tegument proteins which are thought to play an important role in the initiation of replication.

The initial viral genes expressed are the immediate early genes. In HSV-1 infections, the virion tegument protein, VP16, which is released into the cell following fusion of the virus to the host plasma membrane (Hancock et al., 2006), binds cellular factors host cell factor (HCF) and octamer DNA-binding protein (Oct-1) which binds to target sequences within immediate early (IE) genes. The VP16 protein stimulates the expression of six IE genes (ICP4, ICP0, ICP22, ICP27, ICP47 and $U_S1.5$) which encode proteins required to transactivate early (E) gene expression and drive the lytic cycle.

Expression of early genes requires the function of the immediate early gene, ICP4. Early proteins include enzymes that are required for the replication of the viral genome and expression of late gene transcription. Late genes are required for the onset of virion viral synthesis. Most viral genes involved in nucleic acid metabolism

are classed as late genes. Late genes are divided into two groups, group1 (leaky-late or early-late) are expressed for a short time in association with the synthesis of immediate-early genes. Group 2 late genes (true-late) are expressed after expression of early genes and are linked to replication. Once expressed, late genes localise in the nucleus and assemble onto the parental DNA to form structures called prereplicative sites. It is at these prereplicative sites that viral DNA synthesis and replication are initiated. Expression of the late genes is increased during replication.

Virus capsid assembly and egress

Capsid proteins are transported to the nucleus of the infected cell, where capsid assembly takes place. Capsids are assembled first containing an internal scaffold protein, which is lost on insertion of the viral DNA. The virus matures and acquires infectivity by budding through the inner lamella of the nuclear membrane, although it is still unclear how the fully enveloped particle is generated. The most recent data suggests that the virion is enveloped at the inner nuclear membrane and then de-enveloped and released into the cytoplasm (Mettenleiter, 2002). The enveloped virus is then transported, through the endoplasmic reticulum and Golgi apparatus, out of the nucleus. Viral membrane proteins are glycosylated as they are synthesised. The mature virion is conveyed to the outer membrane of the host cell inside vesicles. The release of progeny virus is followed by cell death.

1.2.7. Latency

One of the unique characteristics of herpesviruses is in their ability to establish latent infection. Latency is defined as a persistent infection characterized by the fact that no infectious virus can be isolated and no viral antigen can be demonstrated in the latently infected cells (Engles and Ackermann, 1996). Following infection, herpesviruses establish lifelong latent infection at a particular cellular location, where either a continuous low level of virus is produced or episodes of reactivation produce virus (Crawford, 2001).

In HSV, latency is established by the entry of the viral genome into the sensory nerve cell nuclei of the peripheral nervous system (Chen *et al.*, 1995; Jones, 2003). The major viral gene products expressed during the latency are the latent-

associated transcripts (LATs), which play an essential role in the down-regulation of lytic gene expression. The major LAT accumulated during latent infection is in the form of a nonpolyadenylated, stable 2kb intron, which can be spliced to give two smaller fragments, 1.5kb and 1.4kb RNA molecules. Two promoter (LAP1 and LAP2) fragments, located upstream of the start site of LAT, direct the expression of LAT in the sensory neurons. LAP1 is essential for LAT expression in the sensory neurons while LAP2, located upstream of the 2kb LAT, promotes expression of LAT during productive infection. LAP1 and LAP2 play complementary roles during viral life cycle. During lytic infection, LAP2 is the major control element while LAP1 is the dominant control element during latent infection (Chen *et al.*, 1995; Jones, 2003).

The exact mechanism by which LATs perform their function is not clearly understood. It is predicted that latency is established in the absence of immediate-early gene expression. The absence of these transcripts would result in the lack of early and late virus gene expression. LATs are complementary to the IE gene ICP0 and are predicted to inhibit ICP0, which results in the reduction of lytic gene expression and therefore reduces virus replication and aids the establishment of latency (Mador *et al.*, 1998). ICP0 is required for IE promoter activation, activation of expression in all classes of viral genes, binds cellular proteins and is required for efficient HSV-1 reactivation from latency (Jones, 2003; Freeman *et al.*, 2005).

Recent data has shown LATS encode miRNAs, which inhibit apoptosis (Gupta et al., 2006). LAT inhibits apoptosis and maintains latency by promoting the survival of infected neurons, contributing to the persistence of infection. By promoting the survival of infected neurons during latency, LATs increase the number of latently infected cells, thus preventing premature death of neurons prior to the release of infectious virus. The mode which regulates this process however remains unclear. To gain an insight into this mechanism, computational analysis was performed and revealed transforming growth factor-β (TGF-β) and SMAD3 as targets of miRNA-LAT. TGF-β is a known inducer of apoptosis and inhibitor of cell growth (Gupta et al., 2006).

Latency lasts for the life of the host. Reactivation from latency may be activated by external stimuli such as immunosuppression, which may stimulate viral gene expression. This reactivation results in the recurrence of virus transmission and

disease (Jones, 2003). Recent studies regarding the association of HSV-1 reactivation and immunosuppression combined with retention of $CD8^+$ T cells suggest $CD8^+$ T cells play an important role in maintaining a latent infection (Freeman *et al.*, 2005). $CD8^+$ T cell function is regulated by the interaction between an epitope comprised of an antigenic peptide, bound to MHC class I molecules on the surface of the target cell. For $CD8^+$ T cells to regulate latency, the target cells would be the neurons, where virus establishes latent infection. Evidence supporting the role of $CD8^+$ T cells in controlling HSV-1 reactivation has been shown in *ex vivo* cultures of latently infected trigeminal ganglia (TG) of mice, where $CD8^+$ T cells present in infected TG were shown to inhibit reactivation in culture (Liu et al., 2000). Latency-reactivation of HSV-1 may be categorised into three stages; establishment, maintenance and reactivation. Establishment of latency involves entry of the viral genome into a sensory neuron. Maintenance of latency may last the lifetime of the host, and involves the continued expression of LAT. It is during this stage that the virus may not be detected by virus isolation methods and PCR. Reactivation is the stimulation of viral replication. Viral expression can be detected in the sensory neurons, and virus can be isolated from the infected animal (Jones, 2003).

1.3. The Gammaherpesviruses

The gammaherpesviruses are significant pathogens in both humans and animal species and may be characterised by their ability to replicate in lymphoblastoid cells *in vitro*, to establish latent infections in lymphoid tissue *in vivo* and to cause lymphoproliferative diseases (Roizman, 1995; Coulter *et al.*, 2001).

Members of this virus family are specific to either T- or B-lymphocytes and establish latent infections in the lymphoid system following an initial viremia that is controlled by the hosts' immune system (Roizman, B., 1996; Flach *et al.*, 2002). Serious disease may result in an immunocompromised host, incapable of controlling the reactivating virus or when the virus infects an organism other than its natural host (Roizman, B., 1996).

1.3.1. Human gammaherpesviruses

Epstein-Barr Virus (EBV)

In the 1950's, a British surgeon, Denis Burkitt, treating children with extranodal lymphomas observed unusual epidemiological and clinical features of a lymphoma. Tony Epstein and colleagues, on obtaining tumour biopsies, identified a herpesvirus of the tumour cells. This herpesvirus differed from all known human herpesviruses. The herpesvirus responsible was later identified by Epstein, Achong and Barr in 1964 (Epstein *et al.*, 1964) and subsequently became the first candidate human tumour virus (Kieff and Rickinson, 2001). EBV can cause a variety of diseases, the most commonly recognised being Burkitt's lymphoma (BL), infectious mononucleosis (IM), nasopharyngeal carcinoma (NPC) and X-linked lymphoproliferative syndrome and Hodgkin's lymphoma (HL).

EBV is a member of the gammaherpesvirus subfamily, and is the only human herpesvirus of the γ_1-herpesvirus genus *Lymphocryptovirus* (LCV). EBV infects B lymphocytes of over 90% of the general population before adulthood (Crawford, 2001). Primary infection results in transient viremia, followed by a rapid immune response. The γ_1 and γ_2-herpesviruses show similarities in structure and gene organisation, and share genes found in most herpesviruses, such as those encoding structural components proteins involved in the replication of viral DNA, nucleotide metabolism, as well as genes specific to gammaherpesviruses. Despite these similarities, antigenic cross-reactivity between EBV and other human herpesviruses is rare.

EBV structure

EBV was the first herpesvirus to have its complete genome cloned and sequenced (Arrand *et al.*, 1981; Baer *et al.*, 1984) The EBV genome was sequenced from a *Bam*HI library, and this is reflected in many EBV gene names, which have a B prefix. Similar to all other herpesviruses, EBV is comprised of a protein core, containing double-stranded DNA (172kbp), a nucleocapsid with approximately 162 capsomeres, a protein tegument layer and an outer envelope (Kieff and Rickinson, 2001). EBV has a range of envelope glycoproteins, including homologues of well-

conserved herpesvirus glycoproteins gH (EBV gp85/BXLF2), gB (gp110/ BALF4), and gM (gp84/113/BBRF3).

The characteristic features of the genome include 0.5kbp reiterated terminal direct repeats at both termini and a reiterated 3kbp internal direct repeats. These divide the genome into unique long (U_L) and unique short (U_S) regions. The unique long and short regions contain imperfect tandem DNA repeats, mainly found within ORFs. The unique long region contains a duplicate region (D_L) at the left end of U_L, which consists of multiple, highly conserved tandem 125bp repeats and 2kbp of unique DNA. The D_L region is homologous to the D_R region, located at the right end of U_L. The D_R region consists of multiple, highly conserved tandem 102bp repeats and a 1kbp of unique DNA. D_L and D_R include the origin for initiation of viral DNA replication in lytic infection. The reiteration frequency of the EBV tandem repeats is variable during viral DNA replication. During EBV infection, the genome becomes an episome with a number of tandem repeats. The EBV genome of the infected progeny cells will have the same number of terminal repeat units as the parent genome, including variation to the parental genome introduced during viral DNA replication.

Two EBV types exist, namely type 1 and type 2. Type 1 is more common in most populations, while type 2 is common in Africa and in people with human immunodeficiency virus (HIV). The main differences between the two types lie in the genes encoding the EBV nuclear antigens (EBNA).

EBV Replication

EBV is transmitted via saliva where it interacts with the host in three distinct ways (i) firstly EBV infects the host's B lymphocytes and induces proliferation of infected cells, (ii) the virus can enter into a latent phase following proliferation and subclinical disease (iii) and finally the virus can become reactivated to produce infectious virus for the reinfection of cells and the transmission of disease (Figure 1.4) (Bornkamm & Hammerschmidt, 2001).

EBV Latency

Three types of latency are distinguished for EBV described in various tumour cell lines and infected B cells, as a result of the RNA synthesised (Ackermann, 2006). In type I (Burkitts' lymphoma), only EBV-encoded nuclear antigen, EBNA-1, and Epstein Barr encoded small RNA (EBERs) are expressed. EBNA-1 has an association with chromosomes which allows the persistence of episomes in EBV-infected cells (Yates *et al.*, 1984). In type II (nasopharyngeal carcinoma), EBERs and EBNA-1 are expressed with the latent membrane proteins LMP-1, LMP-2A and LMP-2B (Gilden *et al.*, 2007). LMP-1 is an oncoprotein which affects multiple cell signalling pathways, contributing to EBV-associated oncogenesis (Chang *et al.*, 2004).

EBV infection of primary B lymphocytes results in type III latency. In addition to the genes seen in Latency II (EBER-1, 2, EBNA-1, LMP-1, 2A and 2B), type III (lymphoproliferative diseases) latency expresses viral nuclear antigens EBNA-3A, 3B, 3C and EBNA-LP, and the highly spliced *Bam* A rightward transcripts (BARTs) which have a variety of roles in the up and down-regulation of viral and cellular gene expression as well as transformation of B cells.

Kaposi's sarcoma herpesvirus (KSHV)

Moritz Kaposi first described Kaposi's sarcoma (KS), in the 1870's, as an aggressive tumour that primarily affected a young age group. Kaposi's sarcoma (KS) is a highly vascularized tumor-like lesion affecting the skin, lymph nodes and viscera, which develops nodular tumors composed predominant of spindle cells (Pyakurel *et al.*, 2007).

Kaposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus-8 was discovered in the search for an infectious agent in KS (Chang *et al.*, 1994) and epidemiological evidence has since demonstrated that KSHV is the causative agent of all forms of KS (Sarid *et al.*, 1999).There are three proliferative diseases associated with KSHV: Kaposi's sarcoma (KS), a tumour of endothelial cells; primary effusion lymphoma (PEL), a B-lymphoma, and a plasma cell form of multicentric castleman's disease (MCD). Castleman's disease is a B-cell lymphoproliferative disorder. All of these tumours are frequently demonstrated in

immunosuppressed patients, and in particular patients with acquired immunodeficiency syndrome (AIDS) (Moore and Chang, 1999). KSHV also occurs in the absence of immunosuppression, for example classical KS tumours are common in elderly Mediterranean and African men (Moore and Chang, 2001).

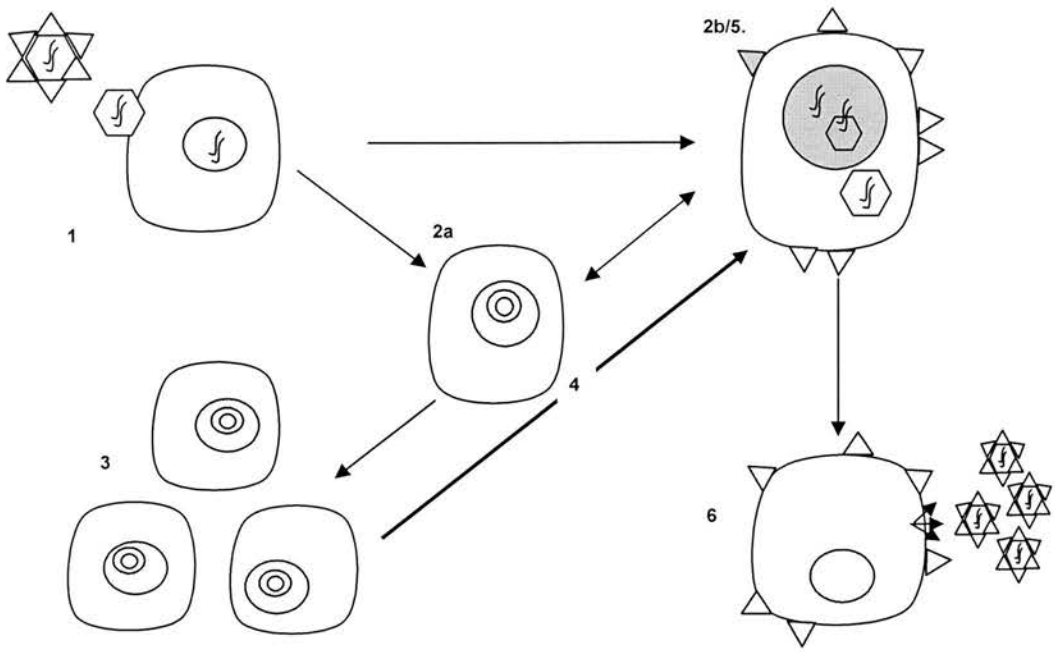


Figure: 1.4: Stages of gammaherpesvirus infected cells (illusion taken from Ackermann *et al.*, 2006). (1) Viral DNA is transported to the nucleus and becomes circularised allowing the cell to become activated. (2) After the infection of B lymphocytes, latency may be established, whereby EBV immobilises the cell, prior to (2a) or following viral replication (2b). Viral genes expressed during latency determine whether infected cells will replicate. The immune response may then be manipulated by the signalled release of host-dependent viral cytokines and chemokines. (4-6) such signalling in the reactivation of latency may result in lysis of the infected cells, subsequently allowing the release of new viral DNA.

1.3.2. Animal Gammaherpesviruses

Gammaherpesviruses of animals are of particular interest, not only as agents of disease, but as models to illustrate the variability and similarity of genome arrangements within members of the gammaherpesviruses subfamily. Animal gammaherpesviruses include equine herpesvirus-2 (EHV-2), equine herpesvirus-5 (EHV-5), bovine herpesvirus-4 (BoHV-4), herpesvirus saimiri (HVS), alcelaphine herpesvirus-1 (AlHV-1) and ovine herpesvirus-2 (OvHV-2).

Herpesvirus saimiri (HVS)

Herpesvirus saimiri (HVS) is the prototype of the herpesvirus genus *Rhadinoviridae* (Fickensher and Fleckenstein, 2001) and is closely related to KSHV. The natural host of HVS is the Squirrel monkey (*Saimiri sciureus*), a native of South American rainforests. The squirrel monkeys are infected via contact with infected saliva, usually within the first two years of life (Fickensher and Fleckenstein, 2001). HVS is asymptomatic in the natural host (Melendrez *et al.*, 1968), while infections of other species of monkey may result in acute peripheral T-cell lymphomas (Schäfer *et al.*, 2003) after experimental infection.

Herpesvirus saimiri was the first rhadinovirus to be completely sequenced (Albrecht *et al.*, 1992) and is known to consist of a 113kb low GC content unique region (34.5%), flanked by about 35 non-coding high GC repeats of 1.4kb (70.8%). The genomes of HVS and EBV are predominantly collinear and homologous genes are in approximately equivalent locations and relative orientations (Albrecht *et al.*, 1992). The genome organization of HVS had also an overall colinearity with that of the gammaherpesviruses BHV-4 (Bublot *et al.*, 2005).

Equine herpesviruses 2 and 5

Five herpesviruses are associated with diseases of horses. Virus has been isolated from horses suffering a range of diseases such as upper respiratory tract disease, perinatal disease, myeloencephalopathy and abortion. EHV-1, EHV-3 and EHV-4 are all alphaherpesviruses, while EHV-2 and EHV-5 are gammaherpesviruses (Telford *et al.*, 1993; Galosi *et al.*, 2005).

EHV-2 was first described in 1962 and was the first gammaherpesvirus to be recovered from horses. The virus causes respiratory disease, including pneumonia in foals and upper respiratory disease in adults. Approximately 80% of horses show serological evidence of EHV-2 infection, as horses carry the gammaherpesvirus in the latent phase of infection for many years, in circulating B lymphocytes (Reviewed by Allen & Murray, 2004). Both viruses are suggested to play a role in upper respiratory tract disease, immune suppression, conjunctivitis and lymphoadenopathy (Telford *et al.*, 1995; Holloway *et al.*, 1999). Diagnosis of the viral agent is difficult; therefore diagnosis is achieved by serological assays. One problem with the use of such tests is the likelihood for cross-reactivity between EHV-2 and EHV-5 viruses due to their close antigenic similarity (Holloway *et al.*, 1999). EHV-5 has only recently been characterised, though little is known regarding the pathogenicity of the disease or the prevalence (Dunowska *et al.*, 1999).The clinical significance of both EHV-2 and EHV-5 remains unknown (Dunowska *et al.*, 2000).

Both EHV-2 and EHV-5 are members of the *gammaherpesvirinae* subfamily, *Rhadinovirus* genus. EHV-2 shows homology to gammaherpesviruses such as HVS (52% homology) and EBV (46% amino acid sequence homology). The linear double-stranded DNA EHV-2 genome is approximately 192kbp and consists of a unique region of 156kbp flanked by a short direct repeat (18kbp). Unique regions in both HVS and EHV-2 are shorter and are flanked by multiple copies of direct repeats; therefore EHV-2 shows a different genome structure to HVS and EBV. Homology is also shown between EHV-2 and other bovine herpesviruses such as bovine herpesvirus-4 (BoHV-4), alcelaphine herpesvirus-1 (AlHV-1) and ovine herpesvirus-2 (OvHV-2) (Telford *et al.*, 1995; Holloway *et al.*, 1999). The distinct EHV-2 and EHV-5 group within the *Rhadinovirus* genus shows a marginally closer association to AlHV-1 than to other viruses in the *Rhadinovirus* genus (Holloway *et al.*, 1999).

Bovine Herpesvirus-4

Bovine herpesvirus-4 (BoHV-4) was first isolated in 1963 in Hungary from calves showing clinical signs of respiratory and ocular disease (Bartha *et al.*, 1966). BoHV-4 is now recognised worldwide, and has been isolated from cattle suffering a

wide variety of diseases; bovine reproductive disorders (Deim *et al.*, 2006) and respiratory diseases, mammary pustular dermatitis (Reed *et al.*, 1977; Li *et al.*, 1991), pneumonia and lymphosarcoma, as well as from seemingly healthy animals (Goyal and Naeem, 1992; Zimmerman *et al.*, 2001). The role of BoHV-4 in the pathogenesis of these diseases however, remains unclear since the virus is found in healthy animals and ones showing clinical disease (Goyal & Naeem, 1992; Chang and Santen, 1992; Izumi *et al*, 2006). Bovine species are recognised as the natural host, however recent studies indicate buffalo as the original host (Dewals *et al.*, 2006) as a survey of wild African buffaloes revealed over 94% had anti-BoHV-4 antibodies (Dewals *et al.*, 2005).

The BoHV-4 genome consists of approximately 145kbp, containing a unique 110kbp region which is flanked by tandem repeats of high G-C content (Bublot *et al.*, 1990). The virus was originally classified as a *betaherpesvirus* due to the formation of inclusion bodies and giant cells after infection of tissue culture cells, characteristic of cytomegalovirus (Storz *et al.*, 1984), as well as the genomic structure (Roizman *et al.*, 1982; Bublot *et al.*, 1990; Bublot *et al.*, 1992). However, further studies on the genome revealed the virus as a *gammaherpesvirus* (Ehlers *et al.*, 1985; Bublot *et al.*, 1992; Lomonte *et al.*, 1995; Lomonte *et al.*, 1996). Its structure, proteins and biology are typical for a gammaherpesvirus (Thiry *et al.*, 1992; Goyal and Naeem, 1992; Egyed, 2000; Fabian and Egyed., 2004).The genomic structure is characteristic of some gammaherpesviruses, typically γ_2-herpesviruses such as HVS (Bornkamm *et al.*, 1976) and γ_1-herpesviruses EBV (Lomonte *et al.*, 1996). The targets for viral replication are the lymphoid organs, the upper respiratory tract and alimentary tracts (Goyal and Naeem, 1992; Asano *et al.*, 2002).

BoHV-4 establishes latent infection in lymphocytes in its natural host (Osorio and Reed, 1983) as well as in experimental hosts such as rabbits (Osorio *et al.*, 1982; Donofrio *et al.*, 2006). It is thought that the site of persistence is a cell of the macrophage/monocyte lineage, while latent infection is established in cells of the immune and nervous systems months later (Donofrio *et al.*, 2001).

BoHV-4 can be transmitted by both horizontal and vertical routes (Donofrio *et al.*, 2000) such as via aerosol droplets and foetal infection (placental tissues).

Virus has also been demonstrated in the cell fraction of milk from infected cattle and it is thought that virus may be transmitted to calves through nursing (Donofrio *et al.*, 2000; Deim *et al.*, 2006). Diagnosis of BoHV-4 is difficult to determine. The virus elicits low-affinity neutralising antibodies which are difficult to identify using neutralisation tests, moreover, in cell culture a cytopathic effect (CPE) is slow to develop (Galik *et al.*, 1993). Detection of BoHV-4 is reliant on serological assays such as indirect-immunofluorescence test, complement fixation and the indirect enzyme-linked immunosorbent assay (Wellinberg *et al.*, 1999). Additional limits to diagnostic tests include the potential for cross-reactivity with other herpesviruses (Osorio *et al.*, 1985). Studies by Rossiter (Rossiter *et al.*, 1989) suggested that a serological antigenic relationship between BoHV-4 and MCF alcelaphine herpesvirus-1 may offer a protective immune response (Dewals *et al.*, 2005). In contrast to this, a survey on BoHV-4 and MCF ovine herpesvirus-2 showed no significant correlation between serological responses to the two viruses (Collins *et al.*, 2000).

1.4. Malignant Catarrhal Fever (MCF)

Malignant catarrhal fever (MCF) is a fatal lymphoproliferative viral disease of cattle, deer and other ungulates (Plowright, 1960; Castro & Daley, 1982; Abu Elzein *et al.*, 2003), whose causative agents are alcelaphine herpesvirus-1 (AlHV-1) or ovine herpesvirus-2 (OvHV-2). AlHV-1, carried by wildebeest, is primarily found in Africa though it has occurred in zoos and animal parks where wildebeest are present (Straver *et al.*, 1979; Hatkin, 1980; Heuschele, 1982).

In addition to the AlHV-1 isolate, other antigenically related viruses have been isolated. AlHV-2 has been isolated from topi and hartebeest (Mushi *et al.*, 1981; Roya *et al.*, 1994). However, AlHV-2 is an apathogenic virus. Experimental infections of cattle AlHV-2 produce no discernible effects and does not generate antibody protection against a subsequent AlHV-1 challenge (Mushi *et al.*, 1981, Reid and Bridgen, 1991; Klieforth *et al.*, 2002). A third distinct gammaherpesvirus was isolated from lymph nodes of roan antelope and was termed HiHV-1 (Reid and Bridgen, 1991). Serology and PCR/sequencing show many more γ_2-herpesviruses in

ungulates (Li *et al.*, 2005). AlHV-1 was initially designated as part of the γ_2-herpesvirus subfamily, due to its biological properties and genome structure (Roizman *et al.*, 1981; Bridgen *et al.*, 1989; Bridgen *et al.*, 1991). This was confirmed when the viral genome was sequenced in 1997 (Ensser *et al.*, 1997). OvHV-2 was defined in Europe as a disease of cattle, which followed contact with sheep. This disease termed "sheep-associated" malignant catarrhal fever, occurs worldwide though is predominately found in Europe, North and South America and New Zealand (Reid *et al.*, 1984). OvHV-2, on the basis of the DNA homology to AlHV-1, is also categorised as a γ_2-herpesvirus (Coulter *et al.*, 2001).

The impact of MCF is difficult to estimate as suspected MCF cases are usually euthanized on diagnostic confirmation. A recent report from the Veterinary Laboratories Agency indicated an increasing incidence of MCF in the UK (Anon, 2006). This increase may simply be due to more effective detection and diagnosis of the disease.

There is an increasing possibility of MCF, particularly that caused by AlHV-1, becoming a more prevalent disease in ranched cattle and other ruminants in countries such as North America subsequent to the increase in wild game ranches, and zoos (Li *et al.*, 1999; Zarnke *et al.*, 2002; Office International des Epizooties, 2005). Infected carriers are asymptomatic, though susceptible animals such as farmed and wild cattle and deer may develop severe and often fatal disease, therefore MCF is of economic and welfare significance worldwide (Plowright *et al*, 1975; Bridgen *et al.*, 1989; Brenner *et al.*, 2002). Studies by Li *et al.*, (1995) and Sausker and Dyer (2002) suggested a low percentage of cattle and deer are seropositive to MCF but display no clinical signs for the disease. A recent survey to evaluate MCF in cattle with no clinical signs (Powers *et al.*, 2005) supported this supposition, showing that OvHV-2 infection can occur without the subsequent development of clinical MCF. Furthermore, subclinical infection is not necessarily associated with the onset of clinical disease (O'Toole *et al.*, 2002; Powers *et al.*, 2005) and some recovered cases were documented.

1.4.1. History of Malignant Catarrhal Fever

Malignant catarrhal fever was initially demonstrated as a disease in Europe in 1789, and was later described by Bugnion in 1877, as a specific, infectious disease affecting cattle (Berkman & Barner, 1958). No cause was originally established, though sheep were implicated as the likely source of infection (Piercy, 1954; Selman *et al.*, 1978; Reid *et al.*, 1984). Numerous attempts to investigate this hypothesis (Piercy, 1954; Pierson *et al.*, 1974) experimentally proved futile, with the exception of studies such as Gotze in 1930 which supported the assumption as cattle were successfully inoculated with MCF (Selman *et al.*, 1978). This finding and subsequent studies confirmed sheep acted as a reservoir host and thus established the term sheep-associated MCF (Plowright, 1964; Buxton, 1980).

A similar disease "snotsiekte", recognised by the Masai tribe in Africa for centuries, was found in cattle (Mettam, 1923; Plowright *et al.*, 1960; Reid *et al.*, 1984). It was determined that this disease occurred when domestic cattle grazed in pastures where wildebeest were calving. The causative agent of this African disease, a herpesvirus, was isolated in 1960 (Plowright *et al.*, 1960).

1.4.2. Hosts

All species of wildebeest, hartebeest and topi are carriers of alcelaphine herpesvirus-1, while domestic and wild sheep are considered reservoir hosts for ovine herpesvirus-2 (Figure 1.5). Furthermore epidemiological evidence indicates the likelihood of goats also being a reservoir for OvHV-2 (Reid 1984). When infected, antibodies develop but no clinical signs are demonstrated. Sheep and wildebeest are therefore asymptomatic carriers of OvHV-2 and AlHV-1.

1.4.3. Host Range

The range of host species susceptible to pathogenic strains of MCF is very wide. MCF occurs primarily in all breeds of domestic cattle, where it equally infects male and female cattle of all ages (Berkman and Barner, 1958). Initially, the highest incidence of MCF was believed to occur between 2 and 5 years of age (Marshall *et al.*, 1920), this however was disproved, and all age groups are considered equally susceptible.

Figure 1.5: Reservoir hosts of MCF. Reservoir hosts of AlHV-1 include all species of Wildebeest (a), Hartebeest (c) and topi (e). Reservoir hosts of OvHV-2 include all species of wild (d) and domestic sheep (b).

Several ruminant species other than cattle are also susceptible to MCF (Figure 1.6). Deer species are particularly susceptible to MCF and it has been recognised in over thirteen species of deer, including: red deer (Reid *et al.*, 1979; Audigé *et al.*, 2001), rusa deer (Tomkins *et al.*, 1997) barbary red deer (Klieforth *et al.*, 2002), fallow deer, white-tailed deer (Li *et al.*, 2000; Li *et al.*, 2003), sika deer (Keel *et al.*, 2003) and axis deer. Pere David's deer (*Elaphurus davidianus*) is reportedly more susceptible to MCF than other species of deer (Reid *et al*, 1987; Orr and Mackintosh, 1988). This is a concern as this species survives only in captivity.

MCF is an increasing concern in zoos, regarding exotic game animals, and in particular captive wild ruminants such as antelope, bison, water buffalo and banteng. Farmed bison herds are similarly susceptible to MCFV, with losses between 33% and 95% in some herds (Schultheiss *et al.*, 2000; Li *et al.*, 2006). Outbreaks in zoos and wildlife parks, however, have led to the identification of novel pathogenic MCF viruses (Flach *et al.*, 2002; Klieforth *et al.*, 2002). The discovery of MCF in pigs in Norway (Loken *et al.*, 1998), Switzerland (Albini *et al.*, 2003), Germany and Finland (Syrjälä *et al.*, 2006) has expanded the range of susceptible hosts of the MCF viruses to include non-ruminant species. Furthermore, animals that have been experimentally infected and found to be MCF susceptible include rabbits (Daubney & Hudson, 1936; Piercy, 1955; Schock and Reid, 1996), hamsters (Reid *et al.*, 1986; Reid *et al.*, 1987), guinea pigs, and rats.

1.4.4. Transmission

The MCF virus associated with wildebeest is predominately cell-associated in adult animals and thus is rarely transmissible. Most wildebeest calves are infected with AlHV-1 before two months of age (Li *et al.*, 2000). The Masai believed that cattle became infected through contact with placenta from a wildebeest (Rossiter, 1983). Although virus has not been detected in the placenta, free virus has been found in nasal and ocular secretions, faecal deposits, and hair follicles of neonatal wildebeest ranging in age from 4 days to 4 months. This has also been demonstrated in adult wildebeest that have been subject to stress (Rossiter, 1983). It is now known that newborn wildebeest are infected during the perinatal period, through horizontal

Figure 1.6: Host Range of MCF. The wide range of host species include all breeds of cattle (f), over thirteen species of deer, including: fallow deer (b), axis deer (c), sika deer (e), white-tailed (g) and Pere David deer (i), and exotic wild and captive animals including: cape buffalo (d), antelope (h), and water buffalo (a), bison (j).

and occasional intrauterine transmission, and are known to shed virus in oculo-nasal secretions until about 3–4 months of age (Mushi *et al.*, 1980; Kim *et al.*, 2003).

Early work by Plowright and colleagues (Plowright *et al.*, 1972) showed some evidence of congenital infection. In the first instance, cows during late pregnancy developed acute MCF while no virus was demonstrated in the foetuses. A further animal gave birth after inapparent infection with MCFV. Thyroid monolayers were prepared from the calf born, and CPE thought to be caused by MCF was observed. The same animal produced a further three calves 80 months post-infection. All calves were shown to be MCF-positive (Plowright *et al.*, 1972).

Recent studies suggest that OvHV-2 transmission within sheep is similar, but not identical, to transmission of AlHV-1 (Kim *et al.*, 2003). With regard to the sheep associated MCF, cases are shown to recur on premises for years, particularly when the sheep or "source" of the virus has not been removed. Most sheep are considered carriers. Furthermore, there is some evidence suggesting that the incidence of MCF in cattle may be higher when ewes are lambing. In contrast to AlHV-1 transmission in newborn wildebeest, the majority of lambs do not appear to be infected until about 2–3 months of age (Li *et al.*, 1998).

Transmission of the two forms of MCF, to susceptible hosts, is most likely a result of inhalation of virus in aerosol droplets. Studies on the transmission of OvHV-2 have shown that the major transmission route is aerosol transmission of nasal secretions from animals suffering virus shedding episodes (Li *et al.*, 2001, 2004). Recent studies by Taus *et al.*, (2006) supported this hypothesis as viral OvHV-2 DNA was detected in nasal secretions in sheep aged between 6 and 9 months. Therefore this study showed that nasal secretions were the predominant mode of transmission as infectivity was demonstrated by inhalation of OvHV-2 secretions into control animals. To study the pathogenesis of the MCF virus, an experimental aerosol infection model was developed in sheep (Taus *et al.*, 2005). Consequently, studies by Taus *et al.*, (2006) showed that nasal secretions collected from sheep were infectious and capable of inducing MCF.

Other transmission mechanisms have also been analysed, including sexual transmission. Studies by Hussy *et al.*, (2002) and Li *et al.*, (2004) regarding the

presence of OvHV-2 DNA in serum and placental/amniotic fluid also indicate a possible minor route of transmission.

1.4.5. Clinical Signs

MCF shows a variety of symptoms, where the course of the MCF infection varies from acute to chronic and is almost invariably fatal (Figure 1.7), though there have been rare reports of recovered cases. It is now thought that infected cattle may show all or no signs of this disease (Reid *et al.*, 1984). This implies that symptomless cases may occur and therefore had previously gone undetected. This also suggested that there may be a higher percentage of animals recovering from this disease than was once thought (O'Toole *et al.*, 1997; Penny, 1998).

Clinical manifestations of MCF are high body temperature, rising to 41-42°C (Selman *et al.*, 1974; Reid *et al.*, 1979), inflammation of conjunctival, nasal and ocular mucosa, leukopenia, lymphadenopathy (Metzler, 1991; Jones *et al.*, 1997), nasal and ocular secretions (Selman *et al.*, 1978; Howard, 1993). Depending on the form of MCF, diarrhoea or constipation may occur. Necrosis and vasulitis in addition to lymphocyte proliferation and tissue infiltration have been demonstrated (Metzler, 1991). Lesions and erosions may be demonstrated in the respiratory and alimentary tract as well as pneumonia, pharyngitis, laryngitis and tracheitis (Florezinek, 1990).

A variety of skin lesions may be observed (Howard, 1993), extensive muscle tremors, incoordination and aggressive behaviour may also be observed.
Clinical MCF can be categorised into four forms. These are:

i. The Peracute form
ii. The Intestinal form
iii. The Head and eye form, and
iv. The Catarrhal form

The peracute form is the most severe of the four categories, where the animal initially exhibits fever, depression and may die within a few hours of these visible symptoms (Reid *et al.*, 1984), commonly due to haemorrhagic gastroenteritis. In

Figure 1.7: Clinical signs of MCF in cattle and Red deer. Characteristic signs of MCF in cattle include bilateral corneal opacity (a, c & e), profuse catarrhal discharge from the eyes and nose (a, e & f) and necrosis of the muzzle (f). Characteristic signs of MCF in deer include catarrhal discharge (b) and diarrhoea (d).

deer, the peracute form, clinical signs may be minimal before death occurs within hours. Clinical signs include a loss of appetite (Reid *et al.*, 1984). The intestinal form, similar to the peracute form, may be characterised by severe enteritis and diarrhoea. Animals commonly die of intestinal MCF within four to nine days. The typical form of MCF, the head and eye form, is characterised by symptoms primarily restricted to the head of the animal. The muzzle epidermis may become encrusted causing an obstruction to the nostrils and may be characterised by open-mouthed breathing and drooling. Nasal discharge is commonly shown to progress from serous to mucopurulent or purulent. Mucopurulent discharge from the ocular mucosa is also common, while progressive corneal opacity is commonly shown in the eyes. The course of this form of MCF, which is invariably fatal, is usually 7 to 18 days. Mild MCF is characteristically associated with experimentally-induced infection with attenuated viruses. This form is generally non-fatal and reports of cattle which are seropositive suggest either an undiagnosed case of MCF or the recovery of an MCF-infected animal (Pierson *et al.*, 1978; O'Toole *et al.*, 1997; Penny, 1998).

1.4.6. Pathology

The severity of gross pathological changes observed in post-mortem examination of an MCF-infected animal may vary depending on the course of the disease, duration of illness and severity of clinical signs. Aside from lesions affecting the external orifices (skin, eyes and mouth), lesions may be shown on a variety of organs and tissue (Figure 1.8) such as; the respiratory tract, lymph nodes, brain, alimentary tract, joints, liver and kidneys (Selman *et al.*, 1974).

The respiratory tract can be severely affected, commonly giving rise to a mucopurulent discharge. Additionally, the larynx, trachea and bronchi can be hyperaemic, with irregular catarrhal accumulations and erosions occurring on the oropharynx and tongue (Selman *et al.*, 1974; Reid *et al.*, 1984; Office des Epizootes, 2005). Ulcers may be located in any area of the oral cavity (Berkman *et al.*, 1960). In general, the involvement of the lymph nodes varies within animals. Lymph nodes may become enlarged, oedematous and may be haemorrhagic and necrotic. Erosions and haemorrhages may be found in the gastrointestinal tract. Changes include extensive haemorrhage and oedema of the intestines, and in particular the bowel. The

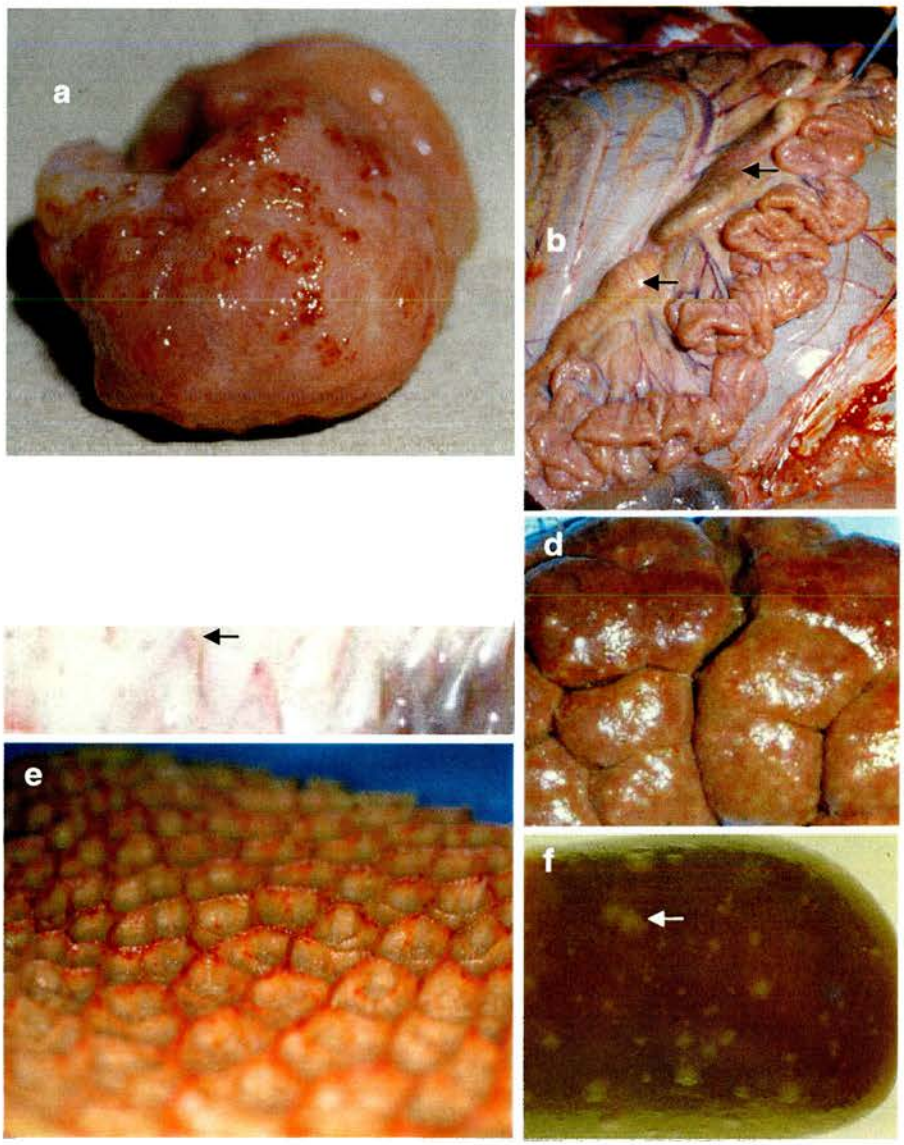


Figure 1.8: MCF gross pathology. Characteristic MCF haemorrhagic lesions shown on a variety of organs such as; the bladder (a), mesenteric lymph nodes (b), buccal pappilae (c), kidney (d, f) and reticulum (e).

abomasum is a common site for congestion, haemorrhage and ulcers (Berkman *et al.*, 1960). The kidneys may become affected with white spots, while haemorrhages may be shown in the liver (Reid *et al.*, 1984).

1.4.7. Histopathology

The microscopic pathology of MCF may be summarised as cellular infiltrates and tissue necrosis (Selman *et al.*, 1974; Liggitt *et al.*, 1978). The main histopathological finding was the widespread accumulation of lymphoid cells around blood vessels (Roberts and Jagger 1961). The lesions demonstrated by MCF infections are characterised by epithelial degeneration, hyperplasia, vasculitis and necrosis of lymphoid organs and the widespread distribution of lymphoid cells in nonlymphoid organs. Organs and tissues mainly affected include the oral and nasal mucosa, gall bladder, urinary bladder, skin, lymph nodes, joints and lungs (Figure 1.9). Infiltrates are localised around blood vessels and are frequently associated with epithelial necrosis and sloughing (Liggit *et al.*, 1978). Pathological changes, such as hyperplasia, are commonly shown in the ileum, caecum and colon. Marked hyperplasia of the lymphocytes may also be seen in all lymph nodes. Lymphocytic vasculitis was detected in the kidney, while the liver showed large numbers of lymphocytes and macrophages particularly around the portal tracts (Roberts and Jagger 1961; Liggit *et al.*, 1978).

No specific tissue has been demonstrated as the site of replication of MCFV in any host. One study demonstrated the presence of virus in nasopharyngeal tissues in sheep, although does not suggest a specific site of entry or replication (Li *et al.*, 2004). Preliminary analysis on diagnostic samples also indicates detectable OvHV-2 DNA in lymph nodes, spleen and kidney (Russell, *per. comm*).

Simple immunoperoxidase staining of samples does not detect viral antigen and classically MCF is regarded as a disease where viral antigen can not be detected. Viral DNA can, however, be detected by PCR. As MCF does not follow a normal herpesvirus lifecycle, it is difficult to predict which antigens would be expressed at a level sufficient to detect protein.

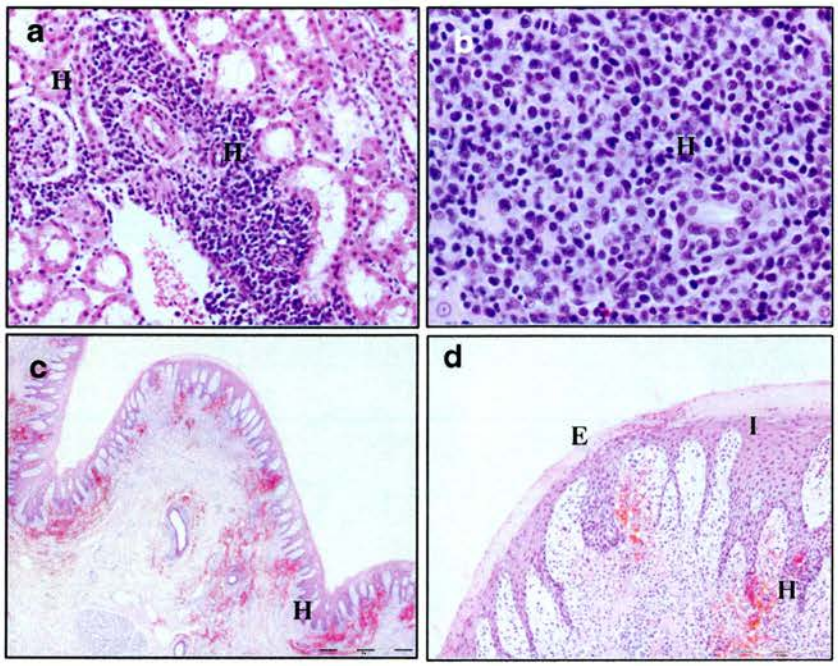


Figure 1.9: Histopathological examination of tissues infected with MCF. H&E staining showing characteristic accumulation of lymphocytes. Cellular infiltrates and tissue necrosis characterised by widespread distribution of lymphoid cells in nonlymphoid organs of a cow experimentally infected with AlHV-1. H&E stained sections include; focal lesions on the (a) kidney, (b) liver, and (a,b) sections of buccal papillae with early MCF lesion. In addition to areas of haemorrhage (H), the stained section shows epidermal erosion (E) and areas of early lymphocyte infiltration (I). All pictures taken at high magnification x 220.

1.4.8. Control

Currently, control of MCF infection is generally based on the separation of susceptible animals from infected carriers such as sheep and wildebeest, particularly around lambing and calving time. This, however, is difficult to achieve in zoos and wildlife parks. Therefore these parks are encouraged to consider introducing only serologically negative animals (Office International des Epizooties, 2005). Treatment of MCF with a range of antiviral and immunosuppressive drugs can be effective (Milne and Reid, 1990) but the duration of treatment and expense make treatment a non-viable option for the control of disease, particularly in developing countries.

Another possibility for control is vaccination. A number of approaches to develop a vaccine have so far been attempted and have failed. Initial attempts to vaccinate cattle involved inoculation of living or inactivated tissue culture grown AlHV-1, combined with incomplete adjuvant. High levels of virus neutralising antibody were detected in these vaccinated animals however no protection against the disease was demonstrated after challenge with virulent AlHV-1. This suggested that humoral immunity on its own was not important in protection against AlHV-1 (Plowright *et al.*, 1975). Rabbits were vaccinated with the inactivated virulent C500 strain of AlHV-1 (Russell, 1980). Four of the six rabbits remained healthy for up to five months after two challenges with the C500 strain. This outcome was encouraging, suggesting the vaccination with inactivated C500 strain AlHV-1 may be of use in protecting cattle. A further study on rabbits, inoculated with inactivated cell-free C500 strain of AlHV-1 in complete Freund's adjuvant showed protection against challenge with virulent cell-free but not cell-associated virus (Edington and Plowright, 1980). The difference in protection between these findings and the previous experiments in cattle may be due to a number of factors such as the virus strain used, the presence of killed Mycobacterium tuberculosis in the adjuvant or the susceptibility of the host animals used.

A subsequent study by Mirangi in 1991 showed that it was indeed possible to protect cattle against the virulent C500 strain of AlHV-1, using a vaccination programme. Protection was not always demonstrated however, and multiple injections of the virus were required to induce protection, which alone risked induction of an MCF-like disease. Cattle inoculated with attenuated or inactivated

MCFV have been resistant to challenge inoculation, but results to date have not been consistent enough to make vaccination practical. (Milne and Reid, 1990).

These studies suggest that a neutralising antibody response was not sufficient for protection, with the possible exception of the cell-free viral challenge shown in rabbits. The immunity shown in the recovered cattle, described by Plowright (1964) may be due to a cell-mediated immune response. The production of an attenuated AlHV-1 strain that remains cell-associated for use as a candidate vaccine has been hampered by the genomic instability of the virus in culture.

The recent cloning of the AlHV-1 genome into a bacterial artificial chromosome (BAC) may allow development of specifically attenuated mutant viruses capable of inducing strong cell-mediated immunity but not disease (Dewals *et al.*, 2006).

1.4.9. Diagnosis

As clinical and gross pathological descriptions of this disease are varied, specific diagnosis originally depended upon the identification of typical histological lesions in tissues (Reid *et al.*, 1979). Malignant catarrhal fever would normally be considered if an animal begins exhibiting typical clinical signs and lesions. A speculative diagnosis may be reinforced if the infected animal has been in contact with sheep, goats, or alcelaphine antelope.

Serological confirmation is now required as the clinical signs alone may indicate numerous diseases. If MCF is suspected, blood samples are sent to a diagnostic laboratory for identification. If the virus to be identified is from a dead animal, blood and tissue specimens; such as spleen, lymph nodes, thyroid glands and lung, should be collected immediately as the virus is inactivated within an hour after death. For AlHV-1, virus isolation can be attempted using foetal bovine or ovine kidney, thyroid and spleen cell cultures, and intraperitoneal inoculation of domestic rabbits or intravenous inoculation of cattle. Virus neutralization, immunofluorescence, or immunoperoxidase identifies viral isolates.

1.4.10. Virus Detection

The advances in molecular and serological diagnostic assays for the detection of malignant catarrhal fever have provided powerful tools for investigating the pathogenesis and epidemiology of MCF (Hsu *et al.*, 1990: Li *et al.*, 2000).

Antibody can be identified from infected animals, however in some cases of acute MCF antibody may not be present resulting in a disadvantage to serological tests. Sensitive and specific PCR assays have been developed for the detection of MCFV.

Characteristically, little viral antigen can be detected within affected animal tissue by tests including immunofluorescence and ultrastructural examination (Patel and Edington, 1980). It is possible, however, to amplify the viral genome by culture or by PCR. Sensitive and specific PCR assays have been developed for the detection of MCFV. Several PCR methods have now been developed which include assays to detect AlHV-1 (Hsu *et al.*, 1990) and OvHV-2 DNA (Baxter *et al.*, 1993).

Based on the molecular cloning of a genomic DNA fragment from the AlHV-1 strain WC11 (Shi *et al.*, 1988), a diagnostic PCR was first developed for the detection of AlHV-1 viral infection (Hsu *et al.*, 1990). The PCR detected picogram amounts of AlHV-1 DNA from a lysate of infected cells and proved a sensitive and specific method. Following the success of this assay, a two-stage nested PCR using different primer sets from an AlHV-1 sequence was developed to amplify both AlHV-1 and AlHV-2 isolates (Katz *et al.*, 1991). Isolation of DNA from bovine samples showed this to be a sensitive assay, detecting 0.01 $TCID_{50}$ of AlHV-1 DNA. These tests were established using experimentally infected animals (Roya *et al.*, 1994), and therefore may not prove sensitive in the diagnosis of clinical cases.

PCR tests have also been designed for the detection of OvHV-2 DNA, using specific primers based on generic PCR primers (Baxter *et al.*, 1993). The assay proved effective in detecting OvHV-2 DNA in peripheral blood leucocytes (PBL) from clinical and experimental cases of MCF. No product was detected from testing target DNA derived from AlHV-1 and BoHV-4, showing the PCR to be specific for OvHV-2 (Baxter *et al.*, 1993). The development of this diagnostic PCR assay, specific for OvHV-2 may improve the accuracy of diagnosis in clinically infected animals (Baxter *et al.*, 1993). These assays provide information regarding

epidemiological aspects of the OvHV-2 virus and its natural ovine host (Baxter *et al.*, 1993; Li *et al.*, 1994; Wiyono *et al.*, 1994; Sausker & Dryer, 2002). The provision of these tests would therefore prove beneficial in developing control measures to determine the MCF carrier status of animals introduced into a new population.

Alternative PCR methods have been sought and developed. These methods include a competitive PCR (cPCR) developed for the quantitative measurement of OvHV-2 DNA (Hua *et al.*, 1999), and a conventional PCR to discriminate between AlHV-1 and OvHV-2 in a nested format (Flach *et al*, 2002). Real-time PCR assays for the quantitative determination of OvHV-2 DNA (Hussy *et al.*, 2001) and AlHV-1 (Traul *et al.*, 2005) have also been developed for analysis of clinical samples.

Disadvantages of conventional PCRs include the risk of contamination resulting in false-positive samples. A quantitative PCR assay was designed for measuring OvHV-2 DNA (Hussy *et al.*, 2001) and compared it to the conventional assay established by Baxter in 1993. Total agreement was shown between the two assays (100%, n = 152) and therefore the real-time PCR was as effective in the accurate determination of OvHV-2 DNA. However in a comparative study between an AlHV-1 nested assay and AlHV-1 Real-time PCR assay (Traul *et al.*, 2005, the nested PCR remained more sensitive (≅1log), although the real-time PCR was specific in the detection of AlHV-1 DNA (described by Li *et al.*, 2000).

To improve study of the epidemiology of AlHV-1, a real-time AlHV-1 PCR has also been developed, targeting AlHV-1 orf3, which encodes a tegument protein (Traul *et al.*, 2005). Comparing this assay to an AlHV-1 nested assay based on the AlHV-1 transactivator gene ORF50 (described by Li *et al.*, 2000), the nested PCR remained more sensitive (≅1log). Although they are not as sensitive as nested PCR, the real-time PCR assays for both AlHV-1 and OvHV-2 offer the potential to quantify levels of viral and cellular DNA. This may offer advantages in vaccination and epidemiology/transmission studies, where viral loads present in tissues, blood or nasal secretions may be examined (Li *et al.*, 2004).

After the initial infection, usually as a lamb, OvHV-2 becomes latent in sheep and may reappear during later life. As OvHV-2 is a member of the subclass Rhadinoviruses, it is lymphotropic indicating the virus resides in B cells. Nested

PCR, if performed on buffy coat DNA should give a PCR positive result, however real-time PCR assays are not sensitive enough to detect virus. The window of positivity for asymptomatic animals remains unknown

The sensitivity of the PCR methods has allowed the monitoring of MCF infection in carrier and susceptible animals, enabling the pathogenesis of the viral disease to be investigated (Baxter *et al.*, 1993). Histopathological examination is the recognised diagnostic procedure to confirm clinical cases of MCF. However, since the PCR assays have proved sensitive and specific, such methods may be established as a new "gold standard" for diagnosis following validation studies between laboratory tests.

1.4.11. Serology

MCF antibody can be detected using virus neutralisation, complement fixation or indirect immunofluorescence. Virus neutralisation and immunofluorescence tests have been employed to ascertain the MCF carrier status of an animal and for the confirmation of MCF in susceptible species (Wan *et al.*, 1988). Of these tests, the virus neutralisation test is the most virus-specific, as antibodies to other bovine herpesviruses may cross-react in tests such as IFAT (i.e. bovine herpesvirus-1, bovine herpesvirus-2 and bovine herpesvirus-4).

Studies have been performed analysing the possible serological cross-reactivity between MCFV and other herpesviruses (Li *et al.*, 1994; Dubuisson *et al.*, 1989; Li *et al.*, 1995). One study by Osorio *et al.*, (1985) showed bovine herpesvirus-1 (BHV-1) and bovine herpesvirus-2 (BHV-2) did not serologically cross-react with other bovine herpesviruses by indirect immunofluorescence. Cross-reactivity has been shown in immunofluorescence serologic assays between MCF AlHV-1 and bovine herpesvirus-4 (Schmitz & Grumbein, 1981; Wan *et al.*, 1988), where the number of seropositive cattle detected by IFAT was always equal to or greater than the number detected by ELISA tests. Furthermore, a recent study by Dewals *et al.*, (2005) showed that BoHV-4 antiserum stained antigens expressed in the nucleus of AlHV-1 infected cells, and indicated a unidirectional relationship as AlHV-1 serum showed no detectable staining of BoHV-4 infected cells.

There are disadvantages to the virus neutralisation tests, for example; the antibody response in clinically affected animals may be limited, with no or low levels of neutralising antibody. Furthermore a cytopathic strain of the alcelaphine hepesvirus-1 virus is employed in the virus neutralisation test, with a significant incubation period of approximately 10 to 12 days (Heuschele and Fletcher, 1984; Wan *et al.*, 1988). This disadvantage results in a significant period before diagnosis can be reached.

Other serological assays, such as complement fixation, immunodiffusion, counter-immunoelectrophoresis or immunoperoxidase have also been employed to measure specific antibodies to AlHV-1 MCF with some degree of success (Rossiter and Jessett, 1980; Rossiter, 1980; Rossiter, 1981). At present, a commercial enzyme-linked immunosorbent assay (ELISA), utilising commercially prepared alcelaphine herpesvirus-1 monoclonal antibody is available for the detection of antibodies against the AlHV-1 strain of MCF (Li *et al.*, 1994, 2001). This commercially available kit, as well as providing a fast, competent method of testing, additionally reduces the risk of possible laboratory-acquired infections during virus growth and errors within the diagnostic laboratory. Furthermore operator errors may be limited due to the utilisation of single serum dilutions, a straightforward protocol and a numerical readout of results. As this ELISA utilises AlHV-1 antigens it may be of limited value for OvHV-2 antibody detection. There is therefore a need to develop an ELISA to identify OvHV-2 specific antibodies in sera that would benefit routine diagnostic, surveillance of MCF cases and for the analysis of the epidemiology of MCF in sheep and cattle.

Serological tests offer economical, rapid and specific methods for the detection of antibodies against MCFV. The simplicity and lack of cross-reactivity of serological assays offer a reliable, low cost method for routine MCFV diagnosis, epidemiological studies and long-term monitoring.

Powers *et al.* (2005) showed by monitoring OvHV-2 infection and clinical signs of MCFV that OvHV-2 infection may occur in cattle without the simultaneous development of clinical MCF. A proportion of cows tested were positive by serology but remained negative by PCR. This may indicate a low level of viral DNA present

in the sample, possibly as a result of latent rather than lytic subclinical infection. It could also indicate that no virus was present in the blood at the time of sampling. Commonly, only animals with MCF, or affected herds, have been tested. Clinical MCF has a high mortality rate, but testing herds shows some animals with viral DNA or antibody but no clinical signs. It may be possible that some animals get infected and recover or do not show clinical signs. Some reports of animals with a chronic form of MCF and recovery exist (Berkman and Barner, 1958; Hamilton, 1990; Milne & Reid, 1990; Baxter *et al.*, 2003; O'Toole *et al.*, 1995 and 1997). In cases such as these chronic non-fatal forms of MCF, recovery with recrudescent disease and complete recovery, the originally reported rate of fatality (90-95%) is lowered (O'Toole *et al.*, 1995; Brenner *et al.*, 2002). O'Toole *et al.*, (1995) suggested a recovery rate of between 20-50%, with 30% developing atypical chronic MCF. A report of an outbreak in Finland similarly reported a 35% rate of recovery (Stenius 1952, cited in Berkman & Barner, 1958). Eleven cattle which had recovered from an MCF-like disease, over a three year period were investigated (O'Toole *et al.*, 1997) using pathology, serological tests (CI-ELISA) and the OvHV-2 PCR for diagnosis (Baxter *et al.*, 1993). Of the animals examined, ten showed clinical signs of acute MCF, ranging from one to four weeks duration. Six animals recovered clinically with the exception of corneal oedema and perforating keratitis. All recovered animals remain persistently infected (Baxter *et al.*, 1993; O'Toole *et al.*, 1997). All animals tested positive by OvHV-2 PCR. The CI-ELISA, however, proved less reliable, with eight positive and three negative animals.

OvHV-2 infections in cattle result in clinical signs within weeks but in some cases may take months to develop. Similar findings (Otter *et al.*, 2002) suggest that cattle may become OvHV-2 infected, develop clinical MCF and recover. In this study, an outbreak of MCF resulted in 12 animals with clinical MCF. Of these animals, one animal, which had demonstrated typical clinical signs of MCF, recovered after 10 days. This study additionally detected OvHV-2 viral DNA and antibody in animals which did not develop clinical disease and suggests silent infections can occur in outbreaks (Otter *et al.*, 2002).

It is of interest to monitor the immune status of both sheep and cattle for MCFV. Sheep have a lifelong antibody to the virus, as they are expected to carry the

virus asymptomatically. In cattle however, since most animals die from the infection there is no opportunity for the immune response of an individual to drop. Therefore the period in which an animal is antibody positive would not have implications in a serological assay.

1.4.12. Unique genes to the MCF viruses, AlHV-1 and OvHV-2.

Of the seventy genes that have been identified in the determination of the DNA sequence of the OvHV-2 genome, twelve genes are considered unique to MCF viruses (Table 1.2). Moreover, three of these twelve genes are unique to the OvHV-2 genome. Several of the open reading frames (ORFs) found within regions of the genome demonstrate significant homology with cellular proteins, suggesting they have been acquired from host DNA during co-evolution of the virus and its host (Coulter *et al.*, 2001). Included are genes with a role in cytokine signalling (IL10 homologue) and regulation of apoptosis (bcl2 homologues). The genes unique to AlHV-1 were designated ORFs A1-A10, while the equivalent OvHV-2 genes were designated ORFs Ov2-Ov10 (Table 1.2).

ORF A1

ORF A1 encodes 97 amino acids and shows no significant sequence or structural homology to any known proteins (Ensser *et al.*, 1997). There is no OvHV-2 homologue to ORF A1.

ORF A2 and ORF Ov2

ORF A2 encodes a spliced protein of 199 amino acids. A2 is positioned equivalent to a transformation-associated gene of HVS strain C though the amino acid sequence shows no homology to the saimiri transformational protein (STP-C) (Jung *et al.*, 1999; Damania *et al.*, 2000). The recent sequencing of the OvHV-2 genome (Hart *et al.*, 2007) shows that Ov2 encodes a protein containing leucine zipper motif (bZIP) and is a homologue to activating transcription factors (ATF) such as cAMP responsive binding element (CREB) and Jun dimerisation proteins. The similarity between AlHV-1 and OvHV-2 is confined to the N-terminal half of the protein, which contains the bZIP motif.

AlHV-1 Genes	OvHV-2 Genes	Function
A1	-	Unknown
A2	**Ov2**	Transcriptional Regulation
-	**Ov2.5**	IL-10 Homologue
A3	**Ov3**	Semaphorin family of cell signalling molecules
-	**Ov3.5**	Unknown, Secreted?
A4	-	Unknown, Secreted?
A4.5	**Ov4.5**	bcl2 family (apoptosis-regulatory)
A5	**Ov5**	G-protein coupled receptors
A6	**Ov6**	Viral transactivator
A7	**Ov7**	Glycoprotein
A8	**Ov8**	Glycoprotein
-	**Ov8.5**	Unknown
A9	**Ov9**	Bcl2 family (apoptosis-regulatory)
A10	**Ov10**	Transcriptional Regulation

Table: 1.2: Unique MCF genes. AlHV-1 and OvHV-2 ORFs and possible functions (based on sequence homologues).

ORF A3 and ORF Ov3

AlHV-1 ORF A3 and Ov3 are homologous to the cellular semaphorin gene family of cell signalling molecules (Ensser and Fleckenstein, 1995), although Ov3 is some 200 amino acids shorter than A3 at the C-terminus (Hart *et al.*, 2007).

Semaphorins are a large gene family of over 20 proteins, and are consequently categorised into eight distinct groups in accordance with their primary structure. Semaphorins have been implicated in many functions, which include tumour suppression, rheumatoid arthritis and immunoregulation. One member of the semaphorin family is expressed on the surface of T and B lymphocytes, natural killer cells (NK), monocytes and neutrophils, and through an association with CD45, a cell surface protein tyrosine phosphatase, is thought to result in the activation of T

lymphocytes. This semaphorin (CD100) also promotes B-cell survival and aggregation (Bougeret *et al.*, 1992).

Furthermore, a recently demonstrated human semaphorin is expressed in lymphoid tissues including lymph nodes, thymus and the spleen (Lange *et al.*, 1998) and shows homology to the viral semaphorins (46% amino acid identity) of AlHV-1. The occurrence of semaphorins in the genomes of herpesviruses and poxviruses implicate semaphorins in viral subversion of the normal immune response immunoregulation (Comeau *et al.*, 1998).

ORF A4

The A4 ORF shows no homology to any recognised sequence and therefore its function remains unknown. Sequence analysis of the 121 amino acid gene product suggests it has an N-terminal signal sequence and so may be exported. Although ORF A4 is positionally homologous to OvHV-2 ORF Ov3.5, the sequences are unrelated. However, A4 and Ov3.5 are of similar sizes and both have N-terminal signal sequences, implying they may serve similar functions (Hart *et al.*, 2007).

ORF A4.5 and ORF Ov4.5

AlHV-1 A4.5 was not initially reported in the sequencing of the AlHV-1 genome (Ensser *et al.*, 1997; Coulter *et al.*, 2001), but was recently added (Mills *et al.*, 2003). ORF A4.5 and Ov4.5 encode proteins of approximately 212 amino acids that share limited similarity with the bcl2 family of apoptosis-regulatory genes and are therefore thought to be involved in the control of cell death. The encoded proteins also show homology to EBV BALF1, a cellular Bcl-2 protein. EBV BALF1 is 0.7 kb in size and encodes a 220-amino-acid protein in a region of early EBV transcripts (Cabras *et al.*, 2005). BALF1 is analogous to the antiapoptotic BCL-2 homologues present in gammaherpesviruses including HSV and KSHV (Bellows *et al.*, 2002).

ORF A5 and ORF Ov5

These genes share limited identity (49% amino acid identity) and appear to encode proteins with seven transmembrane helical domains (Ensser *et al.*, 1997). This arrangement is characteristic of G-protein coupled receptors (GPCRs). GPCRs

are a protein family of transmembrane receptors that transduce an extracellular, ligand binding signal into an intracellular signal termed G-protein activation. The GPCRs are the largest protein family known, and are involved in all types of stimulus-response pathways Palczewski *et al.*, 2000).

Positional homologues to A5 and Ov5 have been found in EBV (BILF1) and EHV-2, and these are also predicted to encode GPCRs. Homologues are not found in any other sequenced gammaherpesviruses and therefore their specific function is still unknown, though they are thought to contribute to viral pathogenesis through interactions with the host's immune system (Coulter *et al.*, 2001).

ORF A6 and ORF Ov6

AlHV-1 ORF A6 and Ov6 encode proteins with weak homology to the EBV BZLF1 viral transactivator (Ensser *et al*,. 1997). The EBV BZLF1 gene encodes a DNA-binding transcriptional activator similar to the AP-1 bZIP family of transcription factors. Expression of this gene results in the activation of gene expression, causing a shift from latent infection to the lytic cycle (Countryman & Miller 1985; Miller, 1990). Furthermore, this gene also functions in viral DNA replication through binding to the lytic replication origin *oriLyt* (Fixman *et al.*, 1992). HHV-8 ORF K8, positionally equivalent to ORF A6 and Ov6, also shows homology to BZFL1. Although positionally equivalent, there is no experimental evidence that A6 and Ov6 are functionally equivalent to BZFL1 (Coulter *et al.*, 2001).

ORF A7 and ORF Ov7

ORF A7 and Ov7 are positional homologues to EBV BZLF2 which encodes a glycoprotein, gp42 Tm region? (Li *et al.*, 1995; Ensser *et al.*, 1997). Gp42 is known to interact with the β_1 domain of the MHC class II protein, encoded by the HLA-DR gene (Spriggs *et al.*, 1996; Coulter *et al.*, 2001). A soluble version of gp42, which does not impede the binding of EBV particles to receptors on B cells, though it is known to block B-cell transformation, and consequently is thought to be involved in viral entry (Li *et al.*, 1997). Evidence to support the assumption of gp42 being involved in viral entry, possibly penetration of the B-cell membrane, was proved in a

study by Wang and Hutt-Fletcher in 1998, which showed a virus that lacked gp42 could bind to B cells though could not infect or transform B lymphocytes. It is still unknown whether A7 and Ov7 play a similar role in MCF virus entry into the cells.

ORF A8 and ORF Ov8

Analysis of the AlHV-1 and OvHV-2 sequences identified multiple splice sites in the Ov7 and Ov8 sequence (Hart *et al.*, 2007). Sequence analysis of the A7/A8 region shows homology with the Ov7/Ov8 region. ORF A8 and Ov8 are positional homologues to the EBV gene (BLLF1) which encodes a glycoprotein, namely gp350/220 (Coulter *et al.*, 2001). Although the MCF genes show a limited sequence homology to this glycoprotein, they demonstrate structural similarities and are therefore also thought to encode glycoproteins (Ensser *et al.*, 1997). Glycoprotein genes found at the same locus on genomes of gammaherpesviruses analysed include the K8.1 gene of HHV-8, which also encodes glycoproteins (Li *et al.*, 1999; Zhu *et al.*, 1999).

ORF A9 and ORF Ov9

The gene products ORF A9 and Ov9, are assumed to play important roles in cell death regulation, as they share limited similarity with the bcl2 family of apoptosis-regulatory genes (Ensser *et al.*, 1997). All gammaherpesviruses have bcl-2 homologues, although none are similar to A9 or Ov9. In EBV, two bcl-2 homologues are encoded, BHRF1 and BALF1 (described in section A4.5/Ov4.5). EBV BHRF1 is a cellular anti-apoptotic protein which is transcribed in both latent and lytic infection as an immediate-early (IE) gene (Cabras *et al.*, 2005; Li *et al.*, 2006b). Although A4.5/Ov4.5 have some similarity to BALF1, no such similarity is observed between A9/Ov9 and BHRF1.

ORF A10 and ORF Ov10

The ORFs A10 and Ov10 appear to encode a glycoprotein which may be involved in the attachment or entry of the virus into the cell. OvHV-2 has little similarity with AlHV-1 ORF10 (Hart *et al.*, 2007).

1.4.13. Genes unique to the OvHV-2 genome

The DNA sequences of both AlHV-1 and OvHV-2 revealed twelve genes unique to the MCF viruses (Figure 1.10), and three further genes which are unique to the OvHV-2 genome (Thonur *et al.*, 2006; Hart *et al.*, 2006). These genes are designated ORF Ov2.5, ORF Ov3.5 and ORF Ov8.5. The 182 amino acid gene Ov2.5 encodes a homologue of cellular interleukin 10 (IL-10) spliced over five exons (Rosbottom, 2003). IL-10 is a regulatory cytokine involved in the control of immune responses. Homologues of ORF Ov2.5 include EBV BCRF-1 (Hsu *et al.*, 1990) and CMV UL111a (Kotenko *et al.*, 2000). Expression in eukaryotic cells has been demonstrated for Ov2.5, where it was shown to be both secreted and functional (D. Haig, J., Stewart, unpublished data). ORF Ov3.5 encodes a protein with a predicted signal peptide sequence. This 163 amino acid gene product shows no homology to any known genes and the function of this protein is still unknown. ORF Ov8.5 encodes a protein of 390 amino acids with unusual amino acid composition. Similar to ORF Ov3.5, ORF Ov8.5 shows no homology to any other gene.

1.5. Project background

Current diagnosis of MCF infection is to date reliant on an indirect immunofluorescence test (IFAT) and a PCR assay. Although there is an ELISA commercially available (Wan *et al.*, 1988, McGuire *et al.*, 2001), it is of limited value in the detection of OvHV-2 antibodies as it was developed utilising alcelaphine herpesvirus-1 (AlHV-1) antigens. The recent determination of the DNA sequence of the OvHV-2 genome (Hart *et al.*, 2007) has allowed about seventy genes to be identified, of which twelve are unique to the MCF viruses and three are unique to OvHV-2. Consequently, these genes may prove good candidates for the development of a specific OvHV-2 detection assay (Table 1.3).

Preliminary studies following a review of the gamma-herpesvirus literature on diagnostic testing demonstrated recombinant OvHV-2 antigens could be expressed *in vitro* and reacted with MCF case sera (Russell, pers commun). No *in*-vivo trials were initiated. The genes were cloned into bacterial expression vectors and the expression of OvHV-2 genes was demonstrated by western blotting analysis. The experiments

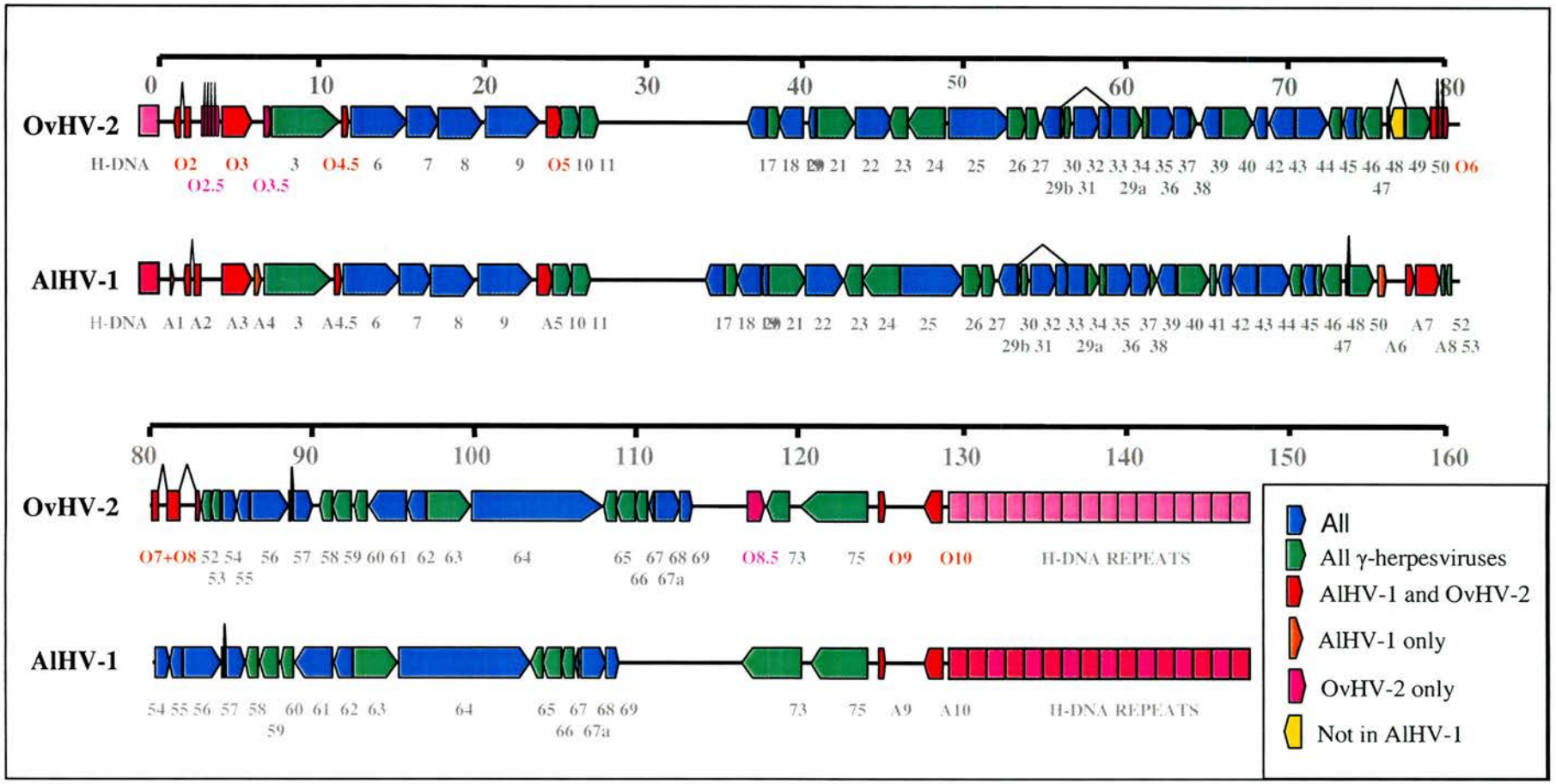


Figure 1.10: Genome organisation of OvHV-2 and AlHV-1. Genes conserved among herpesviruses and gammaherpesviruses are highlighted in blue and green. Genes unique to MCF are highlighted in red. Genes unique to AlHV-1 are highlighted orange while OvHV-2 unique genes are highlighted pink.

also suggested that these genes could be detected by MCF sera, providing reagents that could be utilised to initiate the current study.

MCF unique Genes	Stage of infectious cycle (Immediate/Early/late)	Abundance (Dominant product on cell surface)
A1	Unknown	Unknown
A2/Ov2	Early	Abundant
Ov2.5	Early	Limited
A3/Ov3	Early	Abundant
Ov3.5	Unknown	Unknown
A4	Unknown	Unknown
Ov4.5	Immediate-Early	Limited
A5/Ov5	Early	Abundant
A6/Ov6	Late	Limited
A7/Ov7	Immediate-Early	Abundant
A8/Ov8	Immediate-Early	Abundant
Ov8.5	Unknown	Unknown
A9/Ov9	Immediate-Early	Limited
A10/Ov10	Early	Abundant

Table: 1.3: Summary of MCF genes, Stating gene kinetics (stage in infectious cycle) and gene abundance during infection.

1.6. Project Aim

Despite the severity of MCF in susceptible animals, little virus or viral antigen can be detected and as a consequence, MCF infections prove difficult to detect (Coulter *et al.*, 2001). Therefore the main aim of this research is to advance the current position in the diagnosis of MCF. Detection of antibodies against OvHV-2 antigens will be examined with the aim of developing an enzyme-linked immunosorbent assay (ELISA). In order to achieve this aim several approaches will be considered, including the expression of OvHV-2 candidate antigens as recombinant proteins for the development of an ELISA test development of a virus-

Chapter Two
Materials and Methods

2.1 Animals

2.1.1. Clinical samples

Serum samples from cattle submitted for MCF testing were used throughout. These included cattle with clear signs of MCF and others with clinical signs only indicative of enteric disease. Differential diagnosis between MCF and other viruses such as BVDV can be made on the basis of MCFV testing. Sera from cattle with clinical symptoms indicative of potential MCF were submitted to the Virus Surveillance Unit at the Moredun Research Institute, for routine testing by IFAT. Clinical evaluation and histopathological testing were carried out to provide a final diagnosis for some samples. All sera were stored at -20°C until required.

2.1.2. Experimental AlHV-1 MCF in cattle

Four experimentally infected animals, immunised with high-pass AlHV-1 strain C500 prior to challenge with virulent AlHV-1 virus, were used to establish an in-house direct immunosorbent assay (termed WC11 ELISA) and in a longitudinal study evaluating the antibody response identified by the WC11-ELISA (protocol described in section 2.6.4.1).

The four cattle derived from two groups, given attenuated AlHV-1 (titre $10^{7.5}$/ml) virus intramuscularly, followed at weeks 2 and 16 by either an AlHV-1/chitosan intranasal boost (animal numbers 200819 and 400821), or a chitosan/medium intranasal boost (animal numbers 400251 and 500815). All animals were then intramuscularly challenged with low pass virulent free AlHV-1 at week 22. Blood samples were collected weekly until clinical signs indicated the animal had MCF, such as a sustained increase in body temperature (>40°C for 2-3 days) or ocular/nasal discharge. Rectal body temperatures and physical examination findings were recorded daily for the initial six weeks from these calves, scoring clinically for loss of appetite and increases in temperature. The calves, housed together, were sampled weekly until 212 days post infection. Following inoculation, blood was collected from all calves weekly for analysis by neutralization assays to detect MCF virus specific antibody in plasma.

This work was performed for a vaccine experiment by the MCF group, Moredun Research Institute. Blood collected was subsequently analysed for the validation of the WC11-ELISA. The protocol for the WC11-ELISA is described in chapter five (Section 5.2.1-5.2.2).

2.1.3. Rabbits

Pathogen-free New Zealand white rabbits (Harlan, UK) were used for sera production. Rabbits were housed in individual cages and supplied with proprietary food and water *ad libitum*. Serum was collected from two rabbits, one of which was immunised with OvHV-2 virus–infected mesenteric lymph node, and the second rabbit was immunised with low-pass AlHV-1 strain C500. Blood was collected at the onset of clinical signs (increased temperature ≥ 40°C). Both animals showed clinical signs and pathology consistent with MCF.

This work was performed by Iris Campbell, MCF group, Moredun Research Institute for RNA extraction from infected lymph node.

2.2. Reference Viruses

2.2.1. MCF Viral strains

The virus used for the production of AlHV-1 MCF ELISA antigen was the high-passage culture virus strain WC11, originally described by Plowright *et al.*, (1965). The virus was initially isolated in primary calf thyroid cultures inoculated with leucocytes from the blood of a wildebeest calf, and has subsequently been passaged in primary bovine turbinate (BT) cells. AlHV-1 MCF ELISA antigen was also prepared from virus strain C500, originally described by Plowright *et al.*, (1975).

The source of virus antigen used for the production of MCF OvHV-2 ELISA was a LGL cell line (Strain BJ1035), derived from a cow naturally infected with OvHV-2 (Schock *et al,*. 1994).

2.2.2. Bovine herpesvirus stains

The virus used for the production of BHV-1, strain 6660, (IBR) was originally described by Nettleton & Sharp, (1980). The virus was initially isolated in primary calf thyroid cultures inoculated with leucocytes from the blood of a calf, and has subsequently been passaged in primary bovine turbinate (BT) cells. The virus used for the production of BHV-2 cell lysate was a field sample submitted in 2005 from a severe BHV-2 outbreak in dairy cattle (05/1299). The virus used for the

production of BoHV-4, V-test strain (Belgium), was originally described by Thiry *et al.*, (1981).

2.2.3. Titration of virus

Virus was diluted from a virus stock 10^{-1} to 10^{-7} in diluent (Iscove's medium 10% v/v FBS) and 25µl added to 4 wells of a 96 well tissue culture plate. Uninoculated control wells were also prepared (25µl diluent). 25µl of diluent was added to every well, including the control wells. 100µl of a BT cell suspension (2 $\times 10^5$ cells/ml) was inoculated into every well of the virus dilutions and control and incubated at 37°C (5% CO_2). Plates were read 5-7 days after setting up, for signs of cell growth and cytopathic effect.

2.2.4. Sucrose gradient purification of AlHV-1 virus (strain WC11)

Four 150cm^2 flasks of BT cells were inoculated with 4mls of cell culture grown WC11 virus (4×10^5 $TCID_{50}$/ml) and incubated at 37°C until 90-100% CPE was observed. The cell-associated virus supernatant was harvested after freeze-thawing at -70°C and clarified by centrifugation at 3000 x g for 10 minutes, and virus particles were pelleted at 23,500 x g for three hours. The pellets were resuspended in TNE buffer (50mM Tris pH7.4, 100mM NaCl & 10mM EDTA) and layered onto a discontinuous sucrose density gradient containing sucrose solutions from 20-55 %. The gradient was then centrifuged at 25,000 x g for 16 hours. 500µl fractions were collected and stored at -70°C.

2.3 Cell Culture

2.3.1. Cell growth and maintenance

Maintenance of OvHV-2 LGL cell lines was based on methods described by Reid *et al.*, 1989. There is currently no productive culture system for OvHV-2, therefore a BJ1035 LGL cell-line from an OvHV-2-infected animal (Shock *et al.*, 1998) was grown in 25cm^2 flasks and maintained in IMDM (Gibco Invitrogen, Paisley, UK) supplemented with 10% heat-inactivated foetal bovine serum, 2mM glutamine, 100 IU/ml penicillin, 100µg/ml streptomycin and 350 IU/ml of IL-2

(Proleukin, Chiron Therapeutics, Emeryville, CA). Medium was routinely replaced twice a week.

Bovine turbinate (BT) cells were grown in 225cm^2 flasks and maintained in IMDM supplemented with 10% heat-inactivated foetal bovine serum, 2mM glutamine, 100 IU/ml penicillin, 100μg/ml streptomycin. A monolayer formed in the flasks after about 3-4 days, at which point they could be subcultured.

Virus was grown in the two different cell lines as OvHV-2 has never been propagated in monolayer culture, despite an incomplete enveloped virus detected by electron microscopy from infected rabbits (Rosbottom *et al.*, 2002). As it has been an established cell-line for the continued growth of AlHV-1, by the VSU laboratory at Moredun Research Institute, BT cells were used to obtain cell-associated virus.

2.3.2. Cell passage

For bovine turbinate (BT) cells, medium was removed from the flask and cells washed twice with PBS (37°C). Cells were removed from the flasks using trypsin (0.25%v/v)/versene (0.02%v/v) incubated at 37°C, in a humidified atmosphere of 5% CO_2 in air for 2 minutes. The flask was gently shaken to remove bound cells and medium containing FBS was added to neutralise the trypsin. Cells were passaged twice a week.

In the case of the OvHV-2 LGL cell line, cells were resuspended in the flasks by pipetting. From a 10ml suspension, 5mls were added to a new flask containing 5mls of IMDM medium supplemented with IL-2, while 5mls of medium was added to the original flask. Flasks were incubated at 37°C, in a humidified atmosphere of 5% CO_2.

2.3.3. Cell growth and viability

A 20μl sample of cells was mixed with 180μl of 0.1% (w/v) nigrosine (Sigma), in PBS. Live and dead cells were counted in a haemocytometer, where dead cells were identified by the presence of nigrosine in the cells. Live cells do not allow the entry of the dye. The percentage viability was calculated (i.e. number of live cells / (number of live + dead cells) x 100).

2.4 Molecular Biology

2.4.1. PCR Amplification and Optimisation

Reactions were typically done in 50µl total volume containing 5µl 10x PCR reaction buffer (PCR buffer for KOD Hot Start DNA polymerase), supplied by enzyme manufacturer, 2µl $MgSO_4$ (1.0mM final concentration), 5µl dNTPs (2mM) (Novagen, Germany), 3µl of each primer (5pmol/µl) obtained from MWG-Biotech, 1µl heat-stable DNA polymerase (1U/µl) and 1µl DNA template (100-500ng genomic BJ1035 DNA for amplification of OvHV-2 genes). Samples were made up to the correct volume using water. The heat-activated ultra-high fidelity KOD Hot Start DNA polymerase (Novagen) was used in these experiments to reduce unwanted incorporation errors and to allow hot-start PCR reactions. Reactions were incubated for 2 minutes at 94°C followed by 30 cycles of denaturing [15 seconds at 94°C] annealing [30 seconds at 60°C] and extension stages [20 seconds/kbp at 72°C]. Reactions were carried out in 0.5ml microcentrifuge tubes using a Hybaid Omingene thermal cycler (Hybaid Ltd).

The melting temperatures (Tm) of the primers used in PCR were between 56°C and 70°C. To establish the optimal annealing temperature for each primer pair, an annealing temperature gradient was used. Annealing temperatures varied depending on the PCR primers used and were calculated using the following formula: [(Number of Gs + Cs) x 4] + [(Number of As + Ts) x 2] = annealing temperature (Suggs *et al.*, 1981), (where G = guanine; C = cytosine; A = adenine; T = thymine). However, any bases that were not homologous to the target sequence, such as added restriction sites, were not included in the above calculation.

To analyse PCR products, 10µl of PCR product was mixed with 2µl of loading buffer (80% (v/v) glycerol, 1x TE Buffer, 0.1% (w/v) bromophenol blue) and analysed on a 1% agarose gel. Amplicon size was estimated by comparison with size standards (Hyperladder I, Bioline).

2.4.2. Fragment Purification

Purification of PCR products was performed for the efficient recovery of clean DNA fragments from amplification reactions. PCR products were purified

using the QIAprep PCR purification kit, in accordance with the manufacturer's directions (QIAprep Miniprep Handbook 03/2002). Bound DNA was eluted in 50μl of EB Buffer (10mM Tris·Cl, pH8.5). Eluted samples were stored at -20°C. 10μl of the eluant was run on a 1% agarose gel to estimate the yield of the purified DNA fragments.

Where multiple PCR products were obtained, or where primer-dimers were seen, the specific product band was purified using the QIAquick Gel Extraction Kit in accordance with the manufacturer's directions (QIAquick Spin Handbook 07/2002). The DNA was eluted in 50μl of buffer EB (10mM Tris·Cl, pH8.5) and stored at -20°C. 10μl of the eluent was run on a 1% agarose gel to confirm the presence of the purified DNA fragments.

2.4.3. Ethanol Precipitation

To concentrate the nucleic acids obtained from gel extraction or PCR purification steps, ethanol precipitation was performed following the protocol of Sambrook *et al.*, (1989). One-tenth volume 3M sodium acetate (pH 5.2) and two volumes of ice-cold ethanol (v/v) were added to each sample, before storing for 30 minutes at -20°C to allow the precipitate to form. To improve yield of small fragments DNA, RNA and nuclease free glycogen (5μl=50μg) was added as carrier to some precipitations. The DNA was recovered by centrifugation at 12000 x g for 10 minutes. The pellet was washed with 500μl of 70% ethanol, air-dried briefly and resuspended in 10μl distilled water.

2.4.4. DNA Ligations

PCR products were ligated in accordance with the manufacturer's directions stated in the pGEM®-T Easy Vector system technical manual (manual No. 042 revised 2003, Promega corporation). Each reaction contained 5μl 2x rapid ligation buffer, 1μl pGEM®-T Easy Vector (50ng), 1μl T4 DNA ligase (3 Weiss units/μl) and up to 3μl PCR product, in a reaction volume of 10μl. Negative control reactions were included to check for self-ligation of the plasmid. Ligation reactions were incubated at room temperature for 1 hour then overnight at 4°C.

2.4.5. Transformation using the pGEM®-T Easy Vector ligation reactions

PCR products ligated into pGEM®-T Easy were transformed into chemically-competent JM109 cells. 50μl of JM109 high efficiency competent cells were mixed with 2μl of ligation or control reactions in a 1.5ml tube. The transformation mix was placed on ice for 20 minutes, then incubated at 42°C for 45 seconds and immediately returned to ice for a further 2 minutes. SOC medium (SOB medium [2% (w/v) Trypone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 2.3mM KCl, 10mM $MgCl_2$] plus 20mM glucose) was added to each transformation, which was then incubated at 37°C for 1.5 hours. Reactions were inoculated onto LB agar plates containing ampicillin (100μg/ml), X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) (20μg/ml) and incubated overnight at 37°C.

2.4.6. Treatment of Blunt-Ended PCR Products

KOD Hot Start DNA polymerase generates blunt-ended fragments. Such PCR fragments are not suitable for direct cloning by TA cloning approaches, but can be modified using an A-tailing procedure with Taq DNA polymerase and ligated into the pGEM®-T Easy Vector. Reactions were performed in 10μl total volume containing 7μl PCR product, 1μl PCR buffer (10×) with 1mM $MgSO_4$, 5 units Taq DNA Polymerase and 1μl of dNTPs. Samples were incubated at 70°C for 30 minutes and then stored at -20°C.

2.4.7. Subcloning into expression vectors

NcoI was used for cloning into all expression vectors. NcoI digestion of plasmid vector was done in 20μl total volume containing 1μl plasmid DNA, 10× Promega buffer D and 1μl (10 units) restriction enzyme NcoI (Promega). Digests were incubated at 37°C for an hour. To prevent self-ligation of the NcoI-digested vector, a de-phosphorylation reaction was performed during the final 30 minutes of the NcoI digestion by the addition of 2μl shrimp alkaline phosphatase (SAP). NcoI

digested, dephosphorylated vector fragments were then gel purified as described in section 2.4.2.

Fragments were ligated into expression vectors pBAD, pET and pTrxFus in accordance with the manufacturer's directions. Each reaction contained 5µl 2x rapid ligation buffer (Promega), 1µl vector (50ng), 1µl T4 DNA ligase (3 Weiss units/µl) and up to 3µl NcoI-digest insert DNA, in a reaction volume of 10µl. Negative control reactions were included to check for self-ligation of the plasmid. Ligation reactions were incubated at room temperature for 1 hour then overnight at 4°C.

2.4.8. Analysis of Transformants

2.4.8.1. Single Colony Analysis

A rapid lysate method was used to analyse plasmids for the presence of an insert. Individual colonies or cells scraped from a bacterial patch were lysed by resuspension in 150µl of SDS lysis buffer (2% (w/v) Ficoll, 1% (w/v) SDS, 0.012% (w/v) bromophenol blue in 1xTAE [40mM Tris•Cl, 20mM CH_3COONa, 1mM EDTA, pH8.2]), containing RNase A (100µg/ml). After 15 minutes at room temperature, lysates were spun at 13,000 x g for 15 minutes to pellet insoluble material. The soluble cell lysate (20-50µl) was analysed on a 0.8% agarose gel.

2.4.8.2. Plasmid Preparation by QIAprep Spin Miniprep

Plasmid DNA was used for the initial screening of cloned DNA fragments. A single bacterial colony was picked and grown in 10ml of LB broth containing 100µg/ml ampicillin at 37°C overnight with shaking. Bacterial cells were pelleted by centrifugation at 13000 x g for 10 minutes at room temperature and plasmid DNA was extracted using a QIAprep spin miniprep kit in accordance with the manufacturer's instructions (Qiaprep Miniprep handbook 03/2002, Qiagen). Briefly, the pelleted cells were resuspended in 250µl of buffer P1 (resuspension buffer containing RNase), followed by 250µl of buffer P2 (lysis buffer) and mixed by inversion. Genomic DNA and proteins were precipitated by the addition of 350µl of buffer N3 (neutralising buffer) mixed by inversion and then centrifuged in a microcentrifuge at 13000 x g for 10 minutes. The supernatant was drawn through a

QIAprep spin column (Qiagen), using a vacuum manifold. Unbound material was discarded, and the column was washed with 0.5ml of buffer PB, to remove any remaining traces of nuclease activity. The column was washed with 0.75ml of buffer PE (containing ethanol), unbound material was discarded and the column was centrifuged at 13000 x g for 1 minute to remove any residual wash buffer and ethanol. Unbound material was discarded and the column was placed into a fresh microcentrifuge tube and DNA was eluted by the addition of 50µl of buffer EB (10mM Tris•Cl, pH 8.5) and centrifuged for 1 minute. DNA was stored at –20°C until required.

2.4.8.3. Sequencing

Nucleotide sequencing of PCR products cloned in pGEM-T Easy was carried out by the Functional Genomics Unit at Moredun Research Institute using a MegaBACE capillary DNA sequencer (Amersham) with universal primers. Sequence data was returned as chromatogram files.

2.4.8.4. Glycerol stocks

Glycerol stocks were made by growing *E. coli* bacterial cultures in 10ml L-broth containing 100µg/ml ampicillin at 37°C overnight with shaking. Bacterial cells were pelleted by centrifugation at 3000 x g for 10 minutes at room temperature, before being resuspended in L-broth with 50% glycerol (v/v). Samples were stored at -70°C.

2.4.8.5. Quantification of nucleic acids

DNA was quantified by spectrophotometry. DNA samples were diluted in distilled water (1:20), and using distilled water as a control, the OD_{260} and OD_{280} were determined using a Cecil CE 2041, 2000 series spectrophotometer. The nucleic acid concentration was determined by using the following formula:

OD_{260} reading of 1 = 50µg/ml double-stranded DNA. The purity of the test sample was estimated using the OD 260:280 ratios. Pure preparations have ratios of approximately 1.8.

2.4.9. Preparation of Competent *E. coli.*

Strains of *E. coli* are regularly used as hosts for cloning and expression vectors for specific gene sequences. Many common *E. coli* hosts are commercially available as competent cells to allow rapid transfer of plasmid between strains. For more specific/specialised applications, such as in expression systems, host genotype requirements may mean that specific strains of *E. coli* must be used and competent cells must be made by standard chemical methods.

The *E. coli* strain LMG194 was used as it is capable of growth on minimal medium allowing repression of pBAD by glucose and induction by addition of arabinose. *E. coli* strain BL21 (DE3) carries the gene encoding T7 RNA polymerase and was used for the expression of the pET-22b vector, which is under control of the *lac* promoter, inducible by IPTG. All expression work for the Thioredoxin expression system was performed in *E. coli* strain GI724. This strain contains the bacteriophage-λ *c*I repressor gene under the control of the *trp* promoter inducible by addition of tryptophan. Additionally GI724 is routinely use for the expression of proteins below 30°C.

Competent cells of strains LMG194 and GI 724 were made as follows:

Single colonies from overnight plates were used to inoculate 10ml volumes of LB media and the cultures were incubated overnight at 37°C (shaking at 225rpm). 2.5ml of overnight culture was used to inoculate 100ml of LB media containing 0.2% glucose and the cultures were incubated at 37°C (shaking at 225rpm) until an OD_{600} of 0.2 was achieved. Cultures were chilled on ice and harvested by centrifugation at 4000 x g for 15 minutes at 4°C. The supernatant was removed and the cell pellet resuspended in 50ml ice-cold 0.1M $CaCl_2$ and was placed on ice for 20 minutes. Cultures were centrifuged at 4000 x g for 15 minutes at 4°C and the pellet resuspended in 1ml ice-cold 0.1M $CaCl_2$ with 10% glycerol. Aliquots of competent cells were snap-frozen and stored at -80°C.

2.4.10. Transformation of chemically-competent *E. coli*

Plasmid DNA was transformed into competent *E. coli* strain as follows: 2µl of each plasmid was added to a sterile 1.5ml microcentrifuge tube on ice. A transformation control was used to measure the transformation efficiency of the competent cells by transformation with 2µl of 0.1ng/µl control plasmid (pUC19). 50µl aliquots of each *E. coli* strain competent cells were mixed with plasmid DNA and the controls. The transformations were then treated as stated for pGEM®-T Easy Vector ligation reactions (as described in section 2.4.5).

2.4.11. Agarose Gel Analysis

DNA was analysed by gel electrophoresis on 1% agarose gels containing 0.5µg/ml (w/v) ethidium bromide (Sigma), in TBE buffer (134mM Tris, 44mM boric acid, 2.6mM EDTA). DNA samples were prepared with 1/10 volume loading buffer (15% (w/v) ficoll type, 1x TE buffer, 0.1% (w/v) bromophenol blue). Electrophoresis was carried out at 100V in a horizontal tank (Bio-Rad) containing TBE buffer. The size of DNA bands was estimated by comparison with DNA mass and size standards (Hyperladder I, Bioline). Bands were visualised by UV transillumination and images were captured using an Alphaimager 2200 (Alpha Innotech).

2.4.12. Restriction Endonuclease Digestion of Plasmid DNA

Reactions were made up to a 20µl total volume containing 1µl (200-1000ng) plasmid DNA, 1µl of selected restriction enzyme (4-10u/µl) and 2µl of 10× specific reaction buffer for the chosen enzyme. The digests were incubated at 37°C for at least 1 hour. Digested samples were run on 0.8% agarose gel for one hour at 100 volts.

2.5 Protein Analysis

2.5.1. Total Protein Estimation

To determine the concentration of protein present in soluble extracts, total protein estimation was done using the bicinchoninic acid (BCA) method (Smith *et al.*, 1985) in accordance with the manufacturer's directions (Pierce and Warriner) for

the micro-scale assay, done in 96-well plates. For each assay a standard curve was constructed using bovine serum albumin, 12.5μg/ml to 2mg/ml. The test was read using a Bio-tek ELx808iu Microplate Reader 562at nm.

2.5.2. Polyacrylamide gel electrophoresis (SDS-PAGE)

Protein extracts were run on 10-12% NuPAGE Bis-Tris or Tris-Glycine polyacrylamide gels using the XCell Surelock™ MiniCell. Both chambers of the MiniCell were filled with 1×SDS running buffer (24mM Tris, 250mM glycine, 0.1% (w/v) SDS) running buffer in distilled water). Samples for analysis were diluted 1:1 in 2 x SDS-PAGE sample buffer (100mM Tris (pH6.8), 4% (w/v) SDS, 4% (v/v) β-Mercaptoethanol, 20% (v/v) glycerol, 0.1% (v/v) bromophenol blue) and heated in a boiling water bath for 10 minutes before loading onto the gel. A sample of 5 – 25μl was then loaded onto the SDS-PAGE gel. The samples included a 5μl sample of broad range pre-stained SDS-PAGE standards (Invitrogen, SeeBlue® Plus2) in order to estimate protein molecular weight relative to the standards. All polyacrylamide gels were run at 200V for 1 hour. Gels were used for Western blotting or were stained using Coomassie blue stain, SimplyBlue™ Safestain or Sypro ruby stain (section 2.5.4).

2.5.3. Staining of SDS-PAGE Gels

Polyacrylamide gels were stained in Coomassie blue stain (0.25% (w/v) Coomassie brilliant blue, 10% (v/v) glacial acetic acid, 50% (v/v) methanol) overnight and de-stained over a period of 4–8 hours in Coomassie destain (50% (v/v) methanol, 10% (v/v) acetic acid) until excess stain had been removed. The gel was soaked in a 6% glycerol solution, and then dried onto 3MM paper using a model SE 1106 slab gel dryer (Hoefer Scientific instruments) under vacuum.

Gels were also stained with SimplyBlue™ SafeStain (Invitrogen) and dried onto clearfilm (40μ pore size) using DryEase® mini-gel drying system (Invitrogen). The gels were washed in 100ml distilled water and stained in Simplyblue for 3 hours. Gels were equilibrated in 35ml gel-dry drying solution (10% Ethanol, 0.5% Methanol and 0.5% Isopropanol) for 5 minutes. The gels were placed between pre-wetted cellophane films, ensuring no air bubbles were trapped between the films and supported in a drying frame. Gels were dried for 12-36 hours. Drying by this method allowed subsequent scanning or photography.

2.5.4. Sypro Ruby Stain

SYPRO Ruby stain detects minimally expressed proteins which less sensitive Coomassie Blue staining techniques are unable to detect. Gels were fixed with 100ml of fix solution (50% methanol, 7% acetic acid) and agitated on a shaker for 30 minutes. The gel was stained overnight with 60ml SYPRO ruby gel stain. To minimise background staining, the gel was washed in wash buffer (10% (v/v) methanol, 7% (v/v) acetic acid) and rinsed in distilled water prior to visualisation using a Molecular Imager FX Pro Plus imager (Bio-Rad).

2.5.5. Sample Preparation

To analyse crude extracts, bacterial cells were resuspended in sample buffer (bis-tris gels 4× LDS, tris-glycine gels 2 × SDS) with 10% NuPAGE reducing agent 0.5M DTT to give an approximate OD_{600} of 25. Samples were lysed and solubilised by heating at 85°C for 5 minutes then centrifuged at 13,000rpm for 10 minutes. To analyse soluble extracts prepared by other lysis methods, equal volumes of extract and 2x sample buffer were mixed.

2.5.6. Western Blotting

Western blotting was performed to evaluate the expression and antigenicity of recombinant proteins expressed in pBAD, pET and pTrxFus extracts. After SDS-PAGE, proteins were transferred to a nitrocellulose membrane using the XCell Surelock™ MiniCell. The apparatus was assembled according to the manufacturer's instructions and the tank filled with transfer buffer (25mM Tris, 250mM Glycine, 20% (v/v) methanol) and transfer carried out at 100V for 2 hours.

Two methods were used for preventing non-specific binding of proteins: membranes were incubated for 1 hour in either 3% non-fat milk in PBST (1× phosphate buffered saline with 0.05%Tween-20) or high-salt buffer (0.5M NaCl, 0.05% Tween-20).

Blots were then incubated with primary antibody (anti-HisTag/Anti-Thio) or antiserum for 1 hour followed by a secondary conjugate (e.g. IgG anti-mouse HRP) which recognizes the primary antibody. Chemiluminescent peroxidase substrate (Pierce SuperSignal® ELISA Femto, Alpha Innotech Chemiglow chemiluminescent substrate) was applied to the membrane prior to exposure to x-ray film for exposures

of 1 second to 10 minutes. The exposed film was developed using an automatic film processor (X-OGRAPH Compact X2).

2.5.7. HisProbe™-HRP Technology

HisProbe™-HRP (Pierce, Rockford, Illinois) is a nickel-activated derivative of horseradish peroxidase (HRP) which allows the direct detection of recombinant polyhistidine-tagged fusion proteins. HisProbe™-HRP, diluted 1/1000 in high salt wash buffer (1×PBS, 0.5M NaCl and 0.05% v/v Tween-20) was used in the same way as a primary antibody. Chemiluminescent substrate (Pierce SuperSignal® West Pico Chemiluminescent Substrate) was applied to the membrane prior to exposure to x-ray film for exposures of 1 second to 10 minutes. The exposed film was developed using an automatic film processor (X-OGRAPH Compact X2).

2.6. Diagnosis

2.6.1. Chemical and Biological Reagents – ELISA Kits

The competitive-inhibition (CI-) ELISA kit was supplied by Veterinary Medical Research & Development Inc., (VMRD, Inc. Pullman, USA). The Bio-X BoHV-4 ELISA was supplied by Bio-X Diagnostics, VMRD, Inc.

2.6.2. Indirect Immunofluorescence analysis (IFAT)

The indirect immunofluorescence analysis assay protocol is a modified version of that described by Rossiter *et al.*, (1977). A bovine turbinate (BT) cell suspension (1×10^7cells/ml) was inoculated with WC11 cell culture grown virus supernatant (4×10^5 $TCID_{50}$/ml) and incubated at 37°C for 1 hour. Uninoculated control cultures were also prepared. Iscove's Modified Dulbecco's Medium (IMDM) with 10% fetal bovine serum (FBS), was added to give a final concentration of 10^5 cells/ml. 300µl of control or infected cell suspension were pipetted onto wells on a microscope slide and incubated at 37°C at 5% CO_2 until ~25% cytopathic effect (CPE) was visible. The medium was removed from the slides, and the cell monolayer washed twice with phosphate buffered saline (PBS). The slides were fixed by immersion in acetone (-20°C) for 10 minutes.

As an initial blocking stage, 50µl of 1/20 horse serum in PBS was added to each well and incubated at 37°C for 30 minutes in a moist chamber. The slides were then washed for 2×10 minutes in PBS and stirred continually, using a magnetic stirrer. 50µl of 1/10 dilution of test and control sera was added to the appropriate well, and incubated at 37°C for 30 minutes in a moist chamber. After washing, 50µl of diluted 1/100 Donkey anti-sheep fluorescein isothiocyanate (FITC) conjugate (The Binding Site Limited, Birmingham, UK) was added to each sample and incubated at 37°C for 30 minutes in a moist chamber. Following a further washing stage, the slides were covered with a large coverslip using buffered glycerol as a mountant. The prepared slides were then examined using a UV fluorescence microscope.

2.6.3. Virus Neutralisation assay

The virus neutralisation assay protocol is a modified version of that described by Mushi and Plowright (1979).

All serum samples were heat inactivated at 56°C for 30 minutes. Starting at a neat concentration, 25µl of test serum was added to four wells per two-fold dilution in tissue culture medium (IMDM) in a 96-well flat-bottomed microtitre plate. 25µl AlHV-1 WC11 virus, calculated to be 100 $TCID_{50}$, was added to each well and incubated for 1 hour at 37°C. 25µl 10-fold diluted WC11 virus was back-titrated as a control. 50µl/well of $3x10^5$cells/ml BT cell suspension were coated onto the plate and incubated in a humidified CO_2 atmosphere at 37°C for 7-10 days before being examined microscopically for CPE. The assay is validated by the back titration of virus, which should give a value of 100 $TCID_{50}$ with a permissible range 30-300, and by the positive and negative control sera.

The test serum results are determined by the Spearman-Kärber method (Grist *et al.*, 1966) as the dilution of serum that neutralised the virus in 50% of the wells (Log TCD_{50} = L – d (S- 0.5) where L = lowest dilution, d = difference between log dilution steps, S = sum of proportions of "positive" tests e.g. cells showing CPE).

2.6.4. Enzyme-linked Immunosorbent Assay

The following diagnostic assays for the detection of MCF and BHV-4 antibodies were used:

2.6.4.1 MCF WC11-ELISA (developed in Chapter Five).

2.6.4.2 CI-ELISA - Malignant Catarrhal Fever Virus Antibody Test Kit (VMRD, Inc. USA)

2.6.4.3 BIO-X BoHV-4 ELISA kit (Bio-X Diagnostics, Belgium).

2.6.4.1. MCF WC11-ELISA

Infected virus antigen (AlHV-1 WC11) was coated onto rows 1, 2, 5 and 6, while control antigen prepared from the BT cell line used to propagate the virus, was coated onto rows 3, 4, 7 and 8 of a M129B microELISA plate (Greiner Bio-one) at an initial concentration of 0.5mg/ml, as determined by BCA protein assay, in carbonate buffer (pH9.6). The plate was incubated overnight at 4°C. The negative control acts to differentiate the specific anti-viral antibody from antibodies directed against antigenic determinants of the cell line used, and therefore aims to reduce the number of false positives and establish background reactivity for each antiserum. Between each stage the plates were washed four times in ELISA fluid wash buffer (PBS plus 0.05% (v/v) Tween-20, [PBST]) using an ELISA plate washer (Ultrawash plus, Dynatech laboratories).

All subsequent dilutions were made in PBST containing 10% (v/v) horse serum (PBSTH). 100µl volumes were used. Test sera were added to duplicate infected and control wells, at a constant dilution of 1/100. Furthermore, a MCF-positive and –negative serum were used as test control. The plate was incubated for 1 hour at 37°C. After washing, conjugate (Rabbit anti-bovine IgG horseradish peroxidase [Sigma, Poole,UK]) was added at a 1/1000 dilution to all wells. The plate was incubated for a further one hour at 37°C, washed, and 100µl chromogen tetramethylbenzidine (TMB) peroxidase substrate 1-component added (KPL Insight Biotechnology Limited, Middlesex, UK). The reaction proceeded at room temperature (22±5°C) for 5 minutes before being stopped by the addition of 100µl

0.18M H_2SO_4. The optical densities were read using a MRX plate reader (DYNEX Technologies, Virginia, USA) with a 450nm filter. Results were calculated as mean corrected optical densities, i.e. mean OD of infected antigen paired samples minus mean OD of control antigen paired samples.

2.6.4.1.2. ELISA antigen preparation - Alcelaphine herpesvirus-1

Four 150cm^2 flasks of BT cells were washed twice with Hanks Balanced Salts Solution (HBSS). Two flasks were inoculated with 4mls (4×10^5 $TCID_{50}$/ml) of cell culture grown virus WC11 supernatant, while the remaining two flasks were inoculated with 4mls of HBSS and used both as a negative control and to prepare the negative coating antigen.

All flasks were incubated for one hour at 37°C, with occasional rocking to ensure even distribution of inoculum. 50mls of IMDM medium supplemented with 10% (v/v) FBS, 1% (w/v) glutamine and 1% (w/v) penicillin with streptomycin (100μg/ml) were added to each flask and the flasks were incubated at 37°C until 90-100% CPE was observed normally after 3-4 days.

Infected and control flasks were handled separately. The medium from the flasks, containing infected and detached cells, was decanted into 50ml centrifuge tubes. 50mls of PBS was added to each flask to wash remaining cells. The medium and wash supernatant were centrifuged at 2000 x g for 10 minutes at 4°C, and the cell pellets were re-suspended in 100mls of PBS and re-centrifuged. The supernatant from each tube was discarded. The cell pellets were re-suspended in 1.5ml of 1% Nonylphenylpolyethylene glycol (Nonidet-P40; NP40) detergent in PBS and placed back into the original cell culture flask, to detach the remaining cells. The flasks were incubated for two hours at 4°C, tilting every 15 minutes to remove attached cells. Lysates were removed from each flask, pooled and clarified at 11,600 x g for 5 minutes at room temperature (22 ± 5°C). Supernatant was removed and the pellet discarded. The supernatant was stored at -70°C until required.

ELISA antigen was also prepared using the AlHV-1 strain C500 by the same method. Inoculum was kindly supplied by the Virus Surveillance Unit, Moredun Research Institute.

2.6.4.2. Malignant Catarrhal Fever Virus Antibody Test Kit

This test allows the detection of antibodies against MCFV using a competitive ELISA technique. If antibodies to MCF are present in a test sample, binding to the MCFV antigen occurs, thus inhibiting subsequent binding of the horseradish peroxidase (HRP)-labelled MCF-specific monoclonal antibody (MAb 15-A). If antibodies to MCFV are absent, competitive inhibition does not occur and the MCF-specific monoclonal antibody binds to the MCFV antigen. Determination of results is measured whereby the intensity of colour in the wells is inversely proportional to the concentration of specific anti-MCFV antibodies.

96-well microELISA plates for this assay, coated with MCFV antigen, were supplied by VMRD, Inc. The first six wells in column one served as controls for the test. The controls were provided with the kit and consisted of a positive control, tested in duplicate (wells 2 and 3), and a negative control, tested in triplicate, (wells 4, 5 & 6). Well 1 served as a blank control where no reagents were added in order to measure background absorbance.

Fifty microlitres of control and test sera (diluted 1:5 with serum/conjugate dilution buffer) were pipetted into wells of the MCFV antigen-coated plate, one well per serum. The plate was then covered and incubated for 1 hour at room temperature (21-25°C). After the 60-minute incubation, the plate was washed. The washing procedure consisted of four washes in ELISA fluid wash (PBS plus 0.1% Tween-20) in the ELISA plate washer (Ultrawash plus, Dynatech laboratories).

Antibody-peroxidase conjugate was then prepared by diluting antibody-peroxidase conjugate (100×) in serum/conjugate dilution buffer. 50µl of diluted conjugate was added to each well, with the exception of the blank control. The plate was covered and incubated at room temperature (21-25°C) for 60 minutes. Following a final washing stage, 100µl of substrate (chromogen tetramethylbenzidine (TMB) was added to every well, except the blank control. The plate was then incubated uncovered at room temperature for 60 minutes. The reaction was stopped by the addition of 100µl of 0.18M sulphuric acid (H_2SO_4). Intensity of colour is inversely proportional to the concentration of specific anti-MCFV antibody in the sample tested.

The cut-off threshold was established by adding 3 SD to the mean OD of a pool of 19 OvHV-2 negative sheep, which equalled 25% inhibition. The optical densities (OD) were measured using a plate reader (MRX model, DYNEX technologies) at 450nm filter. The results were expressed as a percentage inhibition, derived by the following formula: **100 – [(mean OD450 of test seum x 100)/(mean OD450 of negative control)]**. For the test to be valid, the mean OD of the negative controls must fall between 0.4 and 2.0, and the positive control must have a percentage inhibition of greater than 25.

2.6.4.3. The BIO-X BoHV-4 ELISA

This test allows the detection of antibodies against BoHV-4 using a direct ELISA technique. If antibodies to BoHV-4 are present, they remain bound to the microwell that contains the viral antigen and the secondary antibody-enzyme conjugate catalyses the transformation of the colourless chromogen into a pigmented compound. The intensity of colour in the wells is proportionate to the concentration of specific BoHV-4 antibodies.

96-well microELISA plates for this assay, coated with BoHV-4 antigen, were supplied by Bio-X Diagnostics. The first well in column one and two serve as positive and negative controls for this test, supplied with the kit. All controls and serum samples had to be diluted 1/100 for the test. 100µl of the diluted sample was then added to the appropriate well and the plate incubated for 60 minutes at 37°C in a humid chamber. After washing, 100µl of conjugate (HRP-labelled anti-bovine IgG monoclonal antibody) was added to each well, and the plate incubated at 37°C in a humid chamber. After the final washing stage, 100µl of the substrate (TMB) was added to each well. The reaction was stopped after 10 minutes of incubation in the dark at room temperature, by addition of 50µl of 1M phosphoric acid (H_3PO_4). Optical densities were measured with a plate reader (MRX model, DYNEX technologies), at a wavelength of 450nm.

The washing procedure consisted of triplicate hand washings, at each stage, with washing buffer provided by Bio-X Diagnostics.

2.6.4.4. Analysis of ELISA results

The ELISA plates were read and the results were expressed as absorbance readings at 450nm. For the WC11-ELISA, if AlHV-1 specific immunoglobulins were present in the test sera, they would be bound by the immobilised AlHV-1 antigen. The secondary antibody binds the antigen-antibody complex and the conjugated enzyme catalysed a colour change proportional to the concentration of specific AlHV-1 antibody in the sample tested. The mean value of the negative virus control was calculated and subtracted from wells containing AlHV-1 antigen to remove background interference.

For the MCF CI-ELISA, the blank control was subtracted from all wells to remove background interference, and the mean value of the negative controls and positive controls were calculated. To obtain the percentage of inhibition, the OD increase of the sample over the mean negative control value was calculated as a percentage, and then subtracted from 100. A positive or negative interpretation was then determined (Table 2.1.).

For the BoHV-4 ELISA, the control (negative antigen) was subtracted from each of the corresponding positive wells. The O.D. signal for each sample was then divided by the positive control serum O.D. and multiplied by 100 to express the result as a percentage (Table 2.2.).

$$100 - \frac{\textit{Sample O.D.}}{\textit{Mean Negative control O.D.}} \times 100 = \textit{\% Inhibition}$$

Percentage Inhibition (%)	Interpretation
< 25	Negative
> 25	Positive

Table 2.1: MCF CI-ELISA. The manufacturers recommended interpretation of results; sample considered positive if yields a result that is greater than or equal to 25% inhibition.

O.D – Mean Neg. × 100 (%)	Interpretation
< 16.35	Negative
16.35 ≤ 59.87	+
59.87 ≤ 103.39	++
103.39 ≤ 146.91	+++
146.91 ≤ 190.43	++++
>190.43	+++++

Table 2.2: Bio-X Diagnositics direct ELISA. The manufacturers recommended interpretation of results, sample considered positive if yields a result that is greater than or equal to one plus sign (+).

2.6.4.5. ELISA Specificity

The potential cross-reactivity of the WC11-ELISA antigen for other herpesviruses of cattle was investigated using the following bovine sera: hyperimmune calf serum produced in a calf immunised with gradient purified infectious bovine herpesvirus-1 (BHV-1 strain 6660, Nettleton & Sharp, 1980). Bovine herpesvirus-4 (BoHV-4) positive serum (BioX Diagnostics, Belgium) and a hyperimmune calf serum produced in a calf intranasally infected with BVDV (strain NADL).

2.6.4.6. Statistical Analysis

Where applicable, standard statistical analysis was carried out through the calculation of standard deviations and mean, using Minitab statistical software (Release 13.1).

Sensitivity and specificity of the MCF ELISA test were measured against one another as well as against the IFAT test, PCR and histopathology/veterinary diagnosis. The proportion of known infected reference animals that tested positive in the assay determined diagnostic sensitivity, while the proportion of uninfected reference animals that tested negative in the assay determined specificity (*Office International des Epizooties*, 2005). All assays were analysed in duplicate.

The Cohen kappa test is used to determine the level of agreement between tests, where no standard test is available. The observed proportion of agreement, *OP*,

between the tests compared is calculated using the total positive and negative samples of each test (Tables 2.3 & 2.4).

$$[OP = (a + d)/n]$$

Where **n** is equal to the total number of samples tested $(n = a + b + c + d)$.

	Test One	
Test Two	**Positive**	**Negative**
Positive	a	b
Negative	c	d

Table 2.3: Determination of the Observed kappa value, *OP*.

The expected proportion of agreement by chance, *EP*, is calculated thus:

$$EP = [\{a + b)/n\} \times \{a + c)/n\}] + [\{c + d)/n\} \times \{b + d)/n\}]$$

Observed agreement – expected agreement = $OP - EP$

The maximum possible agreement beyond chance may be calculated, by subtracting the expected agreement value from one (**1 – *EP***). Kappa is the excess agreement over that expected by chance, divided by the potential excess:

$$kappa = (OP - EP) / (1\text{-}EP)$$

Level of Agreement	Kappa range
Poor Agreement	0
Slight Agreement	0- 0.20
Fair Agreement	0.21- 0.40
Moderate Agreement	0.41-0.60
Substantial Agreement	0.61 - 0.80
Almost perfect Agreement	0.81-1.0
Complete Agreement	1.0

Table 2.4: Determination of *kappa* value. Levels of agreement as demonstrated by Everitt, 1989.

2.7. Antigen Identification

2.7.1. Slide-A-Lyzer® Dialysis system

Dialysis was performed in a 3ml Slide-A-lyzer dialysis cassette (Pierce) with 3mls of experimental and field serum samples. The Slide-A-Lyzer Dialysis Cassette was used for buffer exchange and desalting. The cassette membrane was composed of low-binding regenerated cellulose with a sealed chamber to maintain the highest possible sample retention. To increase flexibility, the membrane was initially hydrated in dialysis buffer (0.1M $NaHPO_4$, 0.5M NaCl, pH 8.3), before loading the sample onto the cassette through a gasket on the cassette using an 18-gauge hypodermic needle. Excess air was then removed from the cassette using the syringe, so the sample contacts a greater surface area. The cassette was floated in constantly moving dialysis buffer, approximately 500 times the volume of the sample, at 4°C for 3 hours. The buffer was then changed, and the sample dialysed overnight. To remove the sample, air is injected into the cassette to separate the membranes, and the sample is withdrawn into a syringe.

2.7.2. Gel Filtration

$Superdex_{200}$ columns (10,000–600,000 globular protein, Average particle size 13μm, flow rate 0.1ml/min) were used for high performance gel filtration of proteins from crude lysates. Gel filtration allows the separation of molecules, dependent on size as they pass through the filtration column. $Superdex_{200}$ columns are produced by the covalent bonding of dextran to highly porous cross-linked agarose beads (Amersham product manual).

Prior to applying the sample, the column was washed using two column volumes of distilled water, followed by one column volume of 0.04% CHAPS in PBS (pH 8.2), to ensure the column was free of the bacteriostat (20% ethanol), present in the packaged column. Crude lysate (0.5mls) was applied directly to the column via the Akta FPLC system. The column was eluted with 0.04% CHAPS in TBS. Fractions (1ml) were eluted and fractions corresponding to protein peaks were analysed by gel electrophoresis and Western blotting, using a HisTag monoclonal antibody (HisProbe-HRP). Samples were stored at -70°C.

2.7.3. HiTrap columns

HisTrap columns are used for the purification of Histidine-tagged recombinant proteins by metal ion affinity chromatography. HisTrap columns consist of cross-linked agarose beads charged by immobilized chelating metal Nickel ions (GE Healthcare Instruction Manual 71-5027-68AE). The column was attached to an Akta FPLC system, and washed with equilibration buffer (PBS), to ensure the column was free of the bacteriostat (20% ethanol), present in the packaged column. Crude extracts of HisTagged proteins, diluted 1:5 in binding buffer (20mM NaH_2PO_4, 0.5mM NaCl, 20mM imidazole under native conditions and 0.1M NaH_2PO_4, 8M urea pH8 under denaturing conditions), and was loaded onto the column. The column was washed extensively with binding buffer and proteins bound to the column were eluted using elution buffer (20mM NaH_2PO_4, 0.5mM NaCl, 500mM Imidazole under native conditions and 0.1M NaH_2PO_4, 8M urea pH4.5 under denaturing conditions). Fractions (1ml) were eluted and fractions corresponding to protein peaks were analysed by gel electrophoresis and Western blotting, using a HisTag monoclonal antibody (HisProbe-HRP). Samples were stored at -70°C.

2.7.4. Sample Preparation

To analyse crude lysate fractions, samples were concentrated 10-fold using Microcon® centrifugal filtration devices with a nominal molecular weight limits (NMWL) of 10,000 (Millipore. Billerica, USA). Concentrated samples were resuspended in SDS sample buffer. Samples were denatured at 85°C for 5 minutes. Equal volumes of sample and 2x sample buffer were mixed. Gels were run at 200v for 1 hour.

Chapter Three
Bacterial Expression Studies

3.1. Introduction

In initial studies performed by a visiting scientist, three OvHV-2 genes were selected for expression after a review of the gamma-herpesvirus literature on diagnostic testing. Most work had involved Human Herpesvirus-8 (Kaposi's sarcoma herpesvirus), where the antigens used diagnostically are the K8.1 glycoprotein, ORF65 (capsid protein) and the bcl2 homologue ORF 16. K8.1 and ORF65 were found to be two of the most antigenic HHV-8 proteins being employed in diagnostic tests such as peptide-based enzyme immunoassays (Perez *et al.*, 2006).

OvHV-2 genes that were positional or sequence homologues (ORF Ov8 (glycoprotein), ORF 65 (capsid protein) and ORF Ov9 (bcl2 homologue) were selected. The genes were cloned into bacterial expression vectors and the expression of OvHV-2 ORF Ov8 and ORF 65 was demonstrated using western blotting with epitope-tag-specific antibodies. There was also some evidence that these genes could be detected by MCF sera, but background was high in the E. *coli* lysates, probably due to cross-reactivity of the sera with bacterial proteins. Nevertheless, these studies provide reagents that were utilised to initiate the current study.

The elucidation of the sequence of the OvHV-2 genome (Hart *et al.*, 2007) enabled the design of PCR primers to amplify selected genes and gene fragments. The gene fragments were given terminal NcoI sites, whereby the central ATG was in frame with the gene of interest, allowing fusion at both ends of the gene. Each PCR fragment was directly cloned into a TA vector, and then inserts with the correct sequence were transferred into expression vectors and transformed into the correct *E. coli* expression host. The vectors chosen were the pBAD/His expression system, the pET-22b expression system and the pTrxFus thioredoxin fusion vector.

The pBAD system is designed for high-level expression of recombinant proteins in *E. coli*, based on the tightly-regulated *araBAD* operon, which is induced by L-arabinose. The pBAD/His series of plasmids are highly inducible expression vectors designed for the regulation of dose-dependent recombinant protein expression and purification in E.*coli*. They are pBR322-derived, allowing the production of soluble recombinant proteins using the *ara*BAD promoter and *ara*C regulator from *E.coli* (Figure 3.1). This controlled promoter–regulator system enables the growth and production of soluble and insoluble proteins in high-cell-density *E. coli* cultures (Lim *et al.*, 2000).When a gene is cloned downstream of the pBAD promoter, expression of the gene is controlled by the *ara*C regulator. The araC

product is a transcriptional regulator, which binds to the O_2 and I_2 half sites of the *araBAD* promoter, in the absence of L-arabinose, forming a DNA loop and blocking transcription. AraC product binding to the O_2 region therefore represses transcription. In the presence of L-arabinose, the *araC* gene product releases the O_2 region and binds to the I_2 region which activates transcription. L-arabinose is therefore essential for expression to occur (figure 3.2). The metal binding region encoded by the histidine tag on the polylinker allows the purification of recombinant protein by methods such as affinity chromatography. pBAD includes a C-terminal 6xHis tag as well as a NcoI site which places the cloned gene at the start of the translated product (Figure 3.1). pBAD only encodes epitope tags for fusion with the gene in question.

The pET system was designed for the cloning and expression of recombinant proteins in *E. coli*. pET is a bacterial system intended for high-level expression of recombinant proteins using the strong phage T7 promoter and E. *coli* strains expressing T7 RNA polymerase, designed to produce large quantities of protein and has several essential components (Figure 3.3).

In 1986, Studier *et al.* developed an advanced expression system, where target genes are cloned under the control of the strong bacteriophage T7 promoter. T7 RNA polymerase is provided from a chromosomal copy of the gene in a specific host (Rosenberg *et al.*, 1987: pET system manual, Seventh Edition). T7 RNA polymerase is highly selective and specific for the T7 promoter. These sequences do not occur naturally in *E.coli* and are not recognized by E.*coli* RNA polymerase.

The target genes, cloned into the pET vector, were transformed into the expression host (BL21) which contains the T7 RNA polymerase gene (λDE3 lysogen) required for protein expression. The T7 RNA polymerase gene is controlled by the *lac* promoter (*lacUV5*). When the *lac* operon inducer, lactose or isopropyl-b-D-thiogalactopyranoside (IPTG), is present, transcription of the T7 RNA polymerase gene is induced. IPTG prevents the *lac* repressor from binding to the *lac* operator allowing transcription to proceed (Figure 3.4). Expression of T7 RNA polymerase at high level following IPTG induction, leads to the strong transcription of genes with T7 promoter sequences and their subsequent overexpression.

A wide variety of pET vectors are available, with a variety of fusion tags and expression signals. The pET-22b vector was selected as it was designed for the fusion of proteins to a signal sequence that facilitates their export to the periplasm.

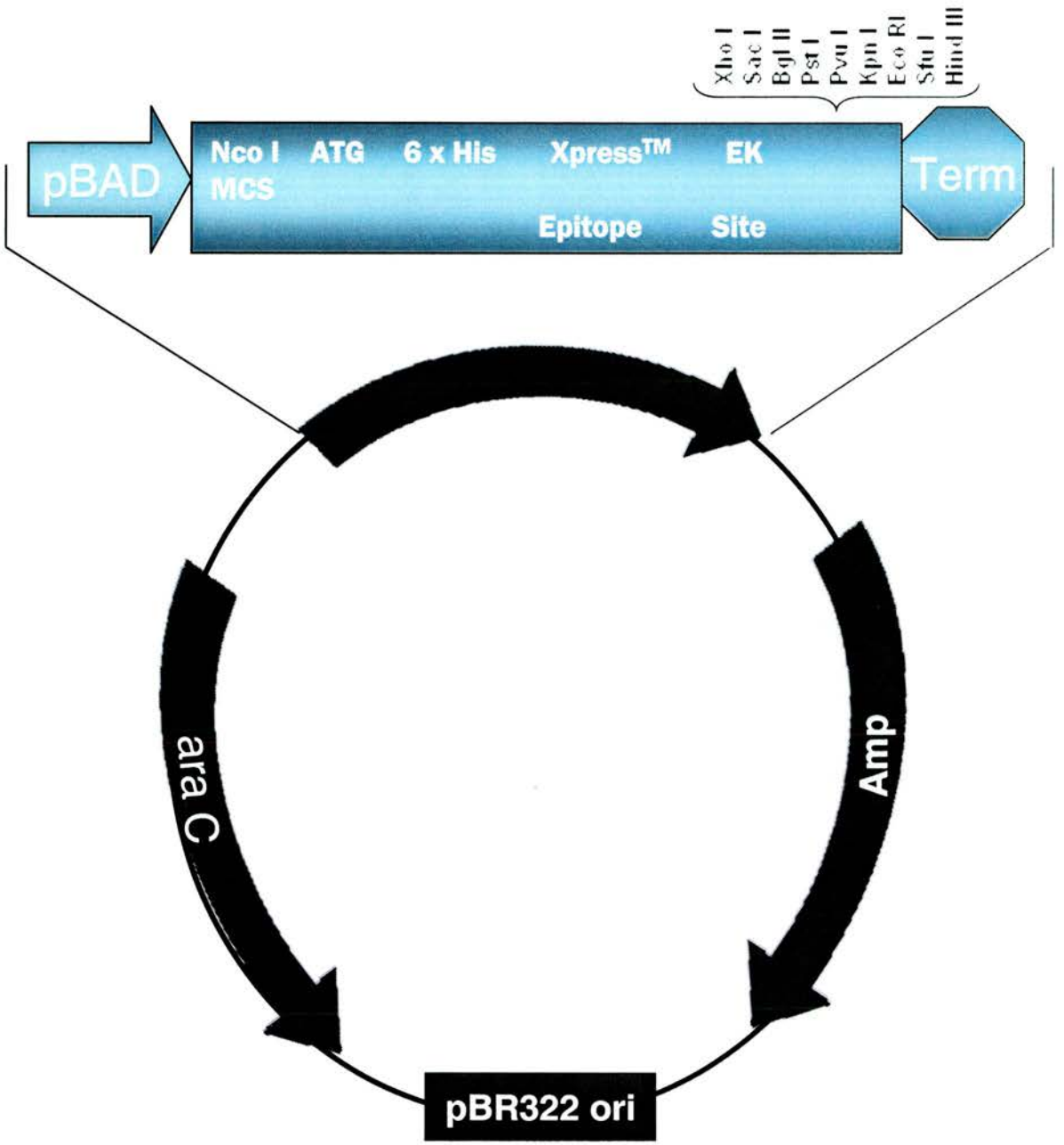


Figure 3.1: The pBAD/His expression system (adapted from Invitrogen). This vector carries a *araBAD* promoter for tightly regulated expression, an enterokinase cleavage site and an N-terminal polyhistidine (6xHis) tag for purification with nickel-chelating resin.

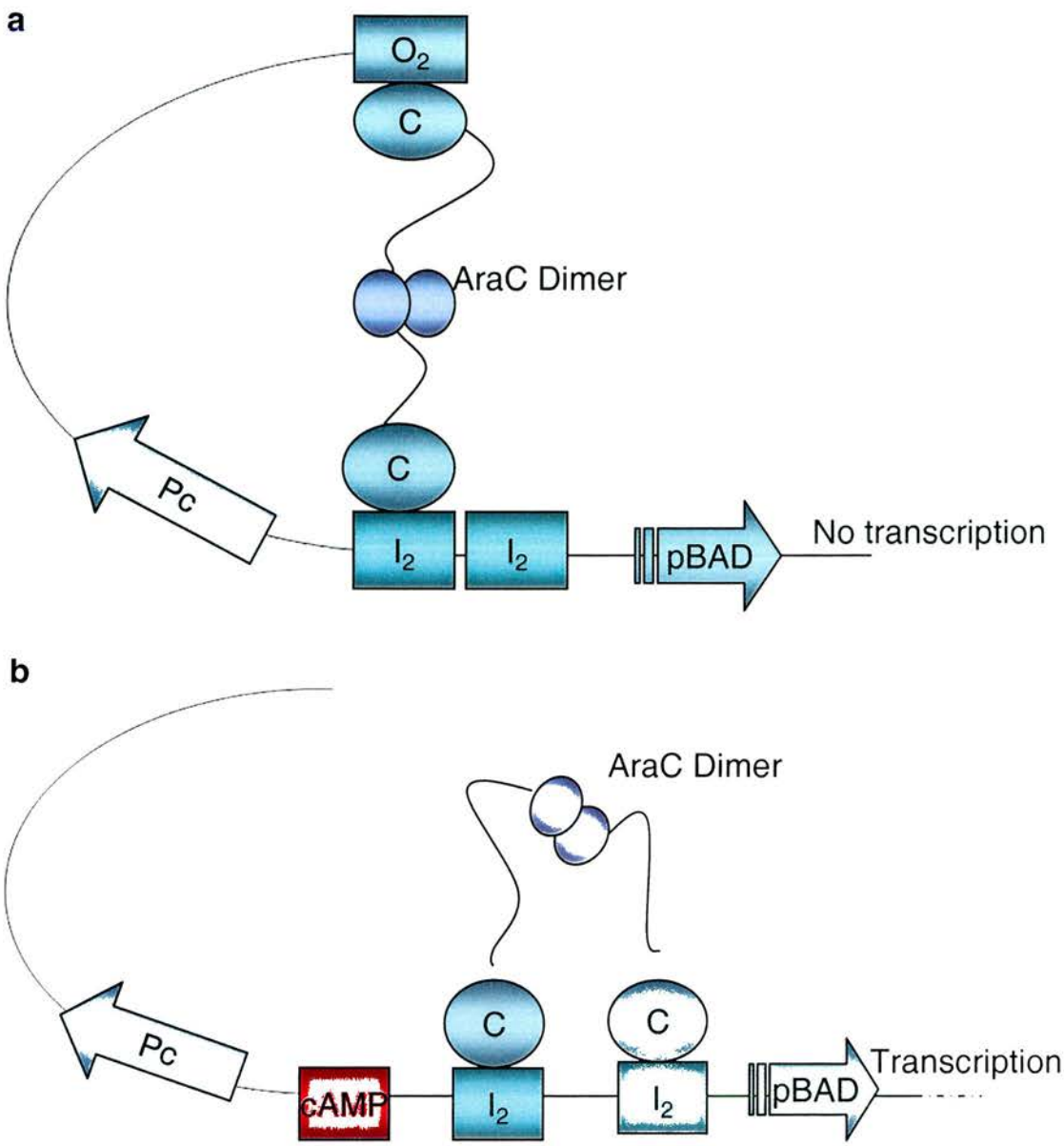


Figure 3.2: Regulation of the araC promoter. a) In the absence of arabinose, the *ara*C dimer binds to O_2 on the *ara*BAD operator, inhibiting transcription. b) In the presence of arabinose, binding to the *araC* occurs and releases the O_2 site, and binds to the I_2 region. The cAMP activator protein binds to the DNA and stimulates the binding of the *ara*C to the I_2 site and allows transcription to occur (Illustration adapted from Invitrogen).

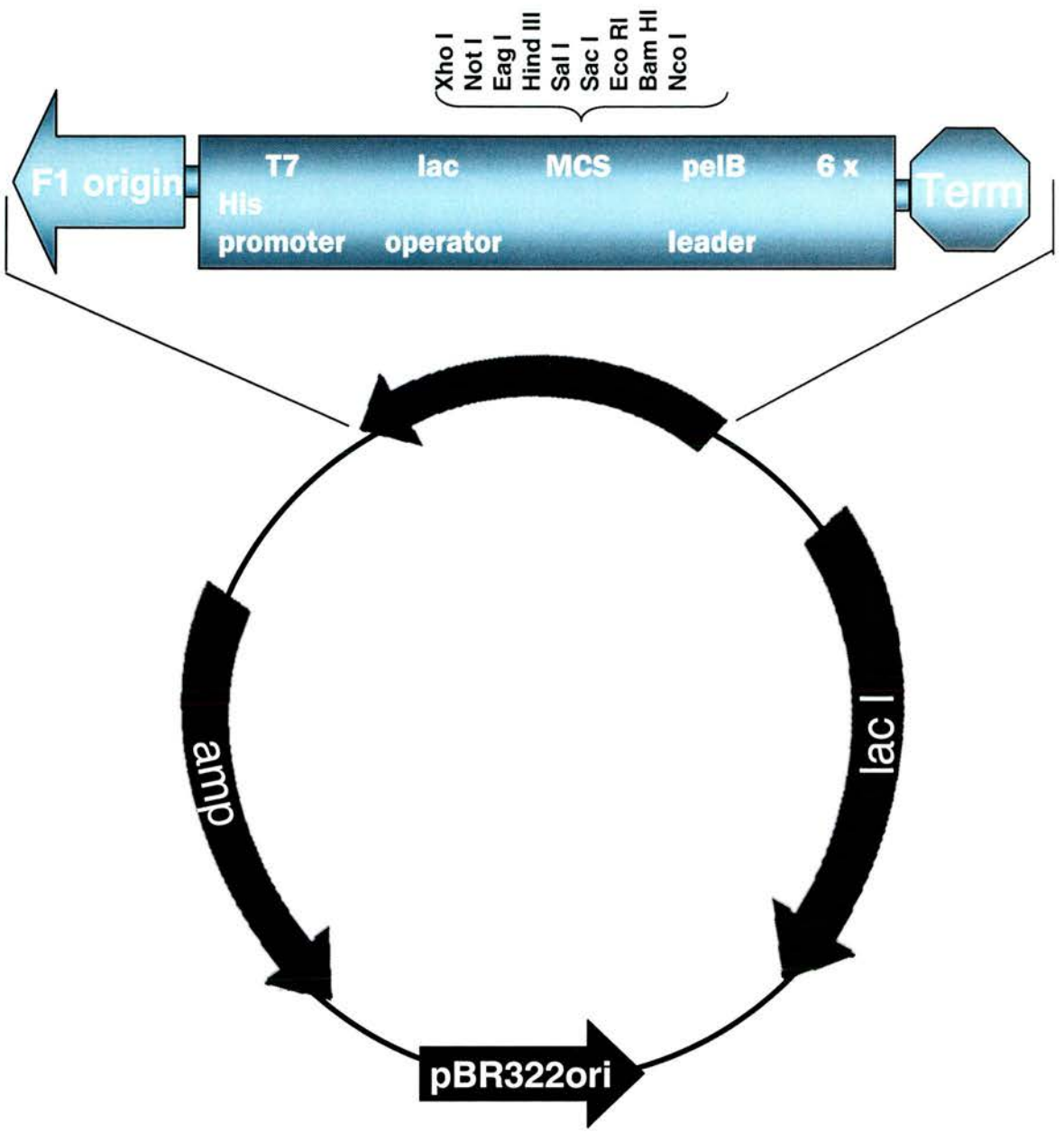


Figure 3.3: The pET vector (Illustration adapted from Novagen). This vector carries a drug resistant marker for ampicillin resistance, the *lacI* gene, the T7 promoter and a polylinker region. The polylinker region contains a pelB signal sequence for potential periplasmic localisation at the N-terminus and a His tag at the C-terminus to aid detection and purification.

Conditions in the periplasm are known to promote proper protein folding and disulphide bond formation and so may enhance the solubility and activity of the target protein (pET system manual, Novagen catalog 2006/2007). The target genes were cloned into the pET-22b polylinker, downstream of the T7 promoter (Figure 3.3). The pET-22b vector carries an N-terminal *pelB* signal sequence which facilitates periplasmic localization and a C-terminal HisTag sequence which aids detection and purification (Figure 3.3). Cloning into the NcoI site places the gene between the signal peptide and the HisTag.

The ThioFusion Expression system was designed to enhance the expression of proteins which have proved insoluble or difficult to express in other bacterial systems. Genes cloned in the ThioFusion expression system vector pTrxFus are expressed as fusions to the *E. coli* protein thioredoxin (trxA) (Figure 3.5). Fusion to thioredoxin can improve the solubility of many eukaryotic proteins expressed in bacteria. In addition, thioredoxin fusions may have unique cellular locations, as thioredoxin localises on Bayer's patches on the cytoplasmic side of the inner membrane (Lunn, *et al.*, 1984), aiding purification by osmotic shock. However, pTrxFus lacked an NcoI site and 6 × HisTag, so to make it compatible with the chosen subcloning approach, and to aid in purification/detection, an oligonucleotide linker (Figure 3.6) was inserted to extend the multiple cloning site and add a C-terminal 6xHis affinity tag (G. Russell, unpublished data).

The plasmid pTrxFus uses a promoter (P_L) from bacteriophage-λ to regulate expression. Expression from pTrxFus is blocked when the bacteriophage-λ *c*I repressor binds to the operator sequence adjacent to the P_L promoter (Figure 3.5). The *c*I repressor gene is present in the chromosome of the *E. coli* cell host (GI 724) and is regulated by the *trp* promoter. For transcription from the P_L promoter to proceed, expression must be induced by the presence of Tryptophan. When *E. coli* GI 724 cells are cultured in tryptophan-free medium, the *c*I repressor is transcribed and binds to the P_L promoter inhibiting transcription. However in the presence of the tryptophan, *c*I repressor synthesis is repressed and transcription from the P_L promoter may occur (Figure 3.7). The target genes, once cloned into the plasmid are transformed into the thioredoxin expression host (GI 724) to allow regulated expression of the fusion protein.

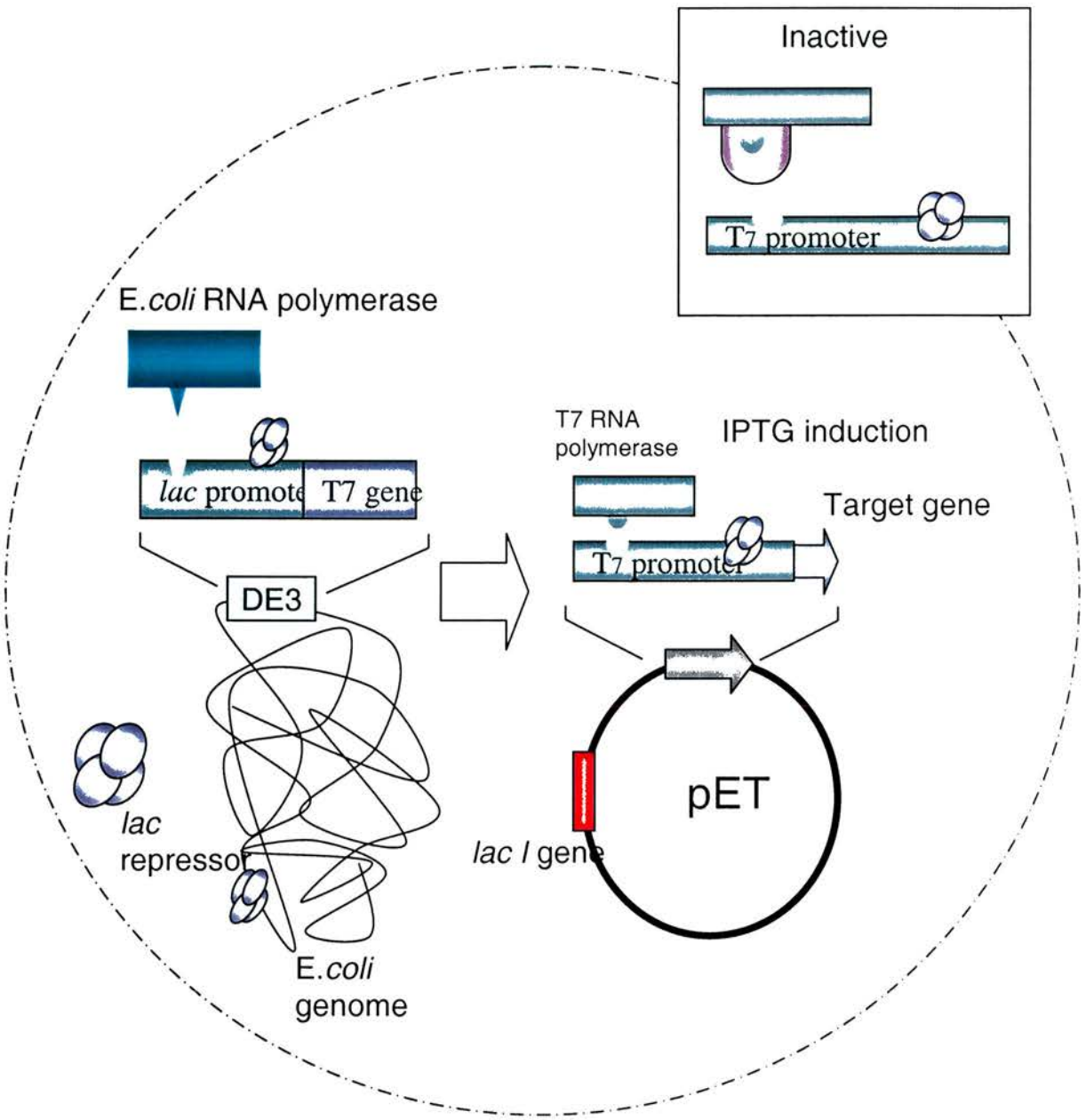


Figure 3.4: Regulation of the *lac* promoter. T7 RNA polymerase is controlled by the lac promoter and regulates transcription. Figure adapted from the pET expression system manual (Illustration adapted from Novagen).

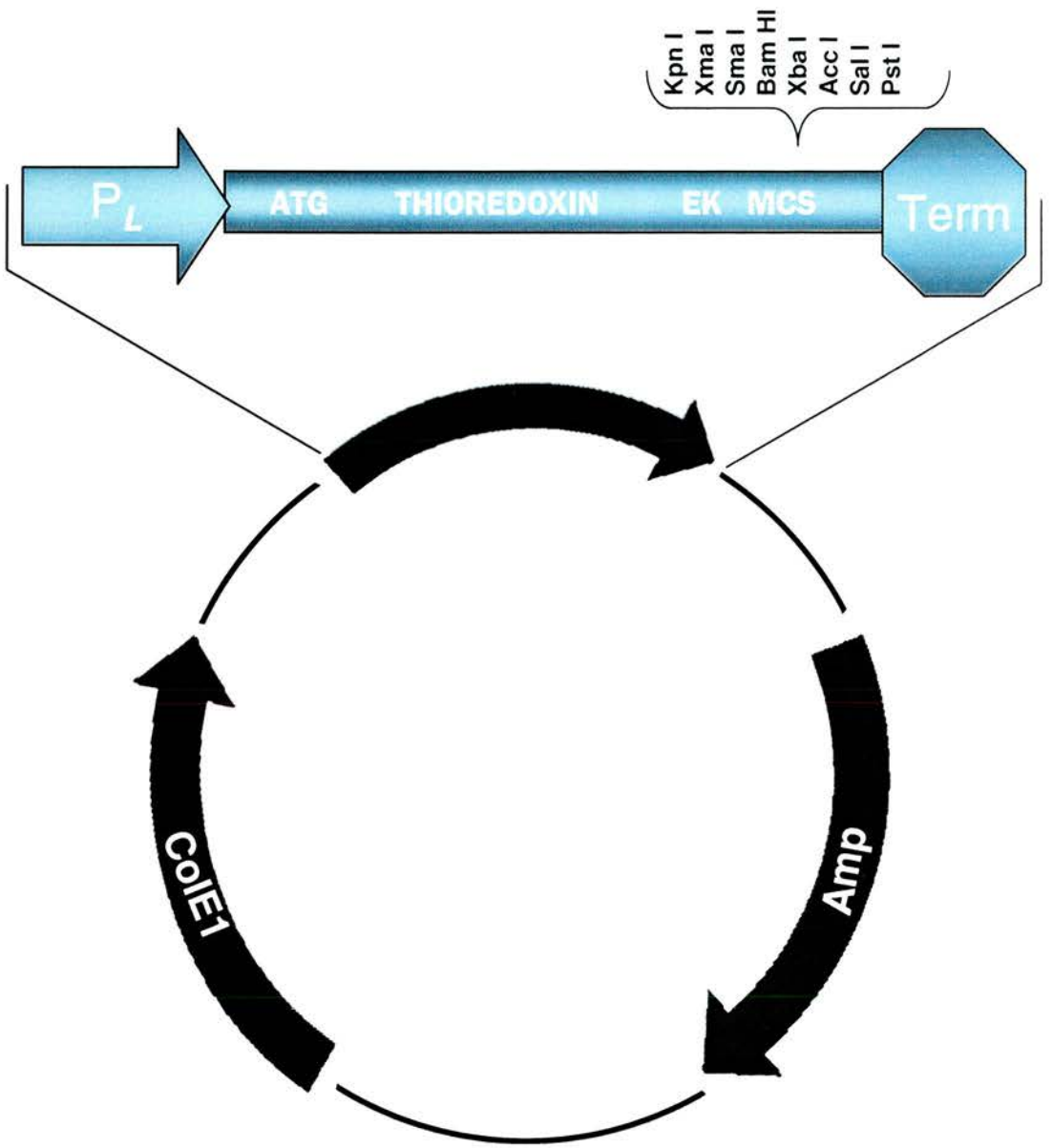


Figure 3.5: The pTrxFus vector for the thioredoxin expression system (Illustration taken from Invitrogen). This vector carries a drug resistant marker for ampicillin resistance, the ColE1 gene, and a polylinker region.

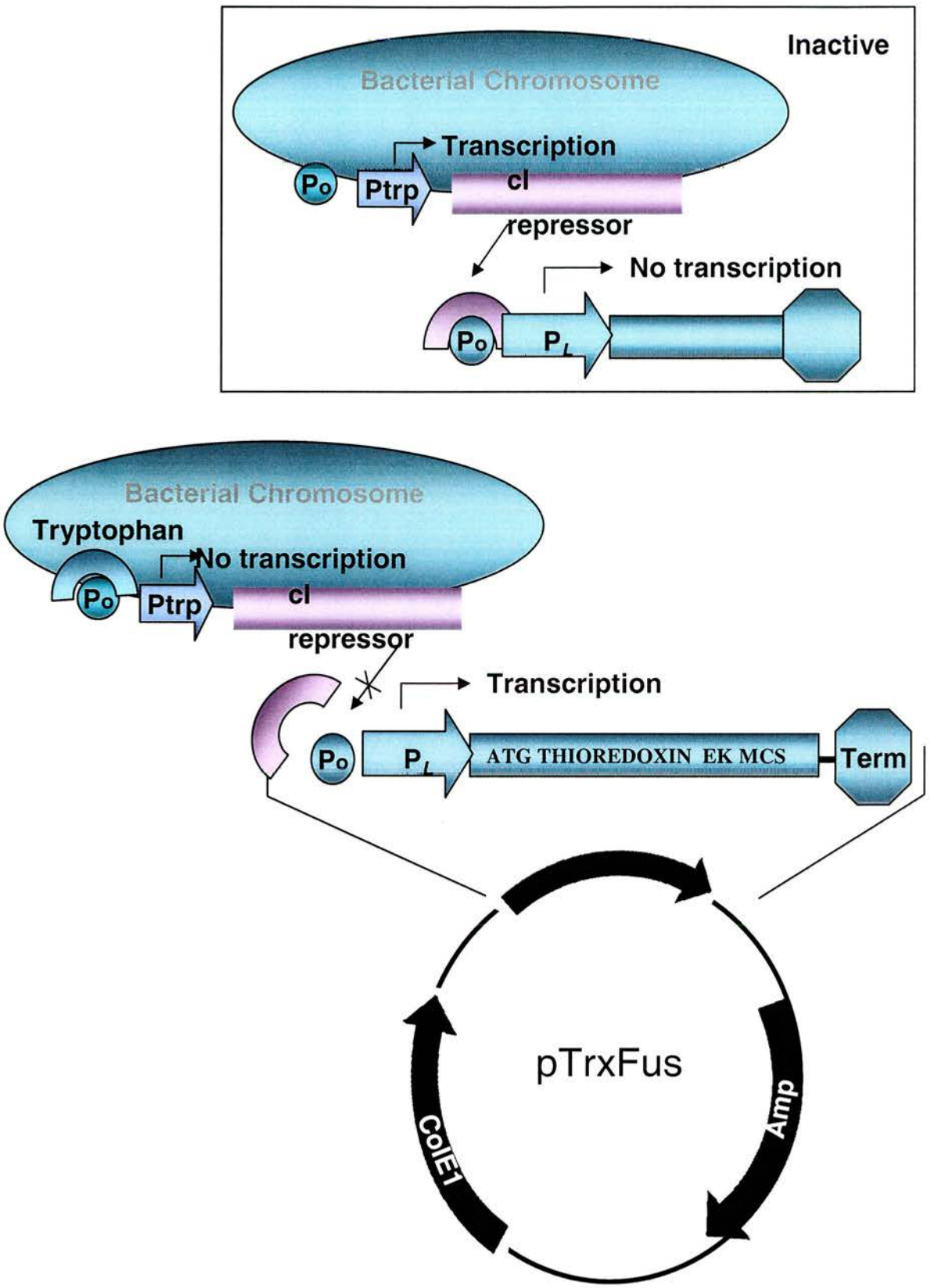


Figure 3.6: Expression of the ThioFusion Expression system, induced by tryptophan. Illustration adapted from Invitrogen ThioFusion Expression System manual.

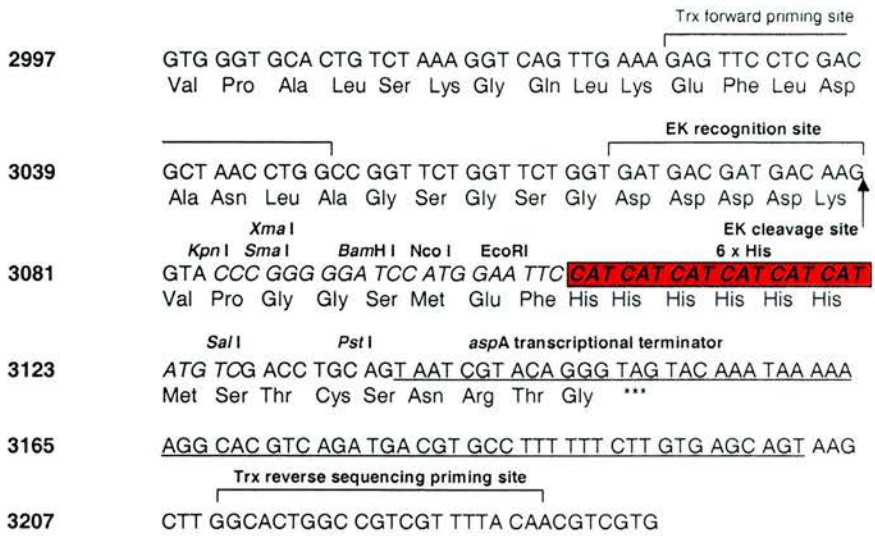


Figure 3.6: 6-HisTag Polylinker. A polylinker designed to add a 6 × His tag to aid purification and detection was added between the XmaI and SalI sites of pTrxFus (shown in italics). The linker also adds Nco I and Eco RI sites for cloning in of amplified OvHV-2 genes

3.1.1 Proteins selected for Expression

OvHV-2 ORF Ov8 is a positional homologue to EBV BLLF1 (gp350/220), KSHV/HHV-8 K8.1 and MHV-68 ORF51, which are known to encode glycoproteins involved in binding to cellular receptors and therefore the Ov8 protein may play a similar role. OvHV-2 Ov8 consists of two exons, with splice sites that are conserved in the homologous ORF A8 of AlHV-1. Analysis of the OvHV-2 sequence identified the presence of multiple potential splice sites in the Ov7 and Ov8 sequence, possibly used to splice the sequences together. Sequence analysis of the Ov7/Ov8 region shows the greatest homology with A7 and A8 of AlHV-1 is located in distinct blocks suggesting a possible significance to gene structure and function. The Ov7 sequence contains a hydrophobic signal sequence but no membrane anchor, while the Ov8 sequence contains a hydrophobic membrane anchor but no obvious signal sequence and therefore suggested the ORFs might form one gene, spliced together to produce one protein product, namely a membrane glycoprotein (J. Rosbottom, PhD thesis, University of Edinburgh 2005).

OvHV-2 ORF Ov9 encodes a protein homologous to the HHV-8 gene ORF 16, which has homology to cellular bcl-2. A number of viruses have been shown to carry apoptosis-inhibiting genes which may play a role in aiding viruses escaping the

host response. Homology between Ov9, ORF16 and members of the bcl-2 family therefore suggests a prolonged life span of persistently infected cells (Neipel *et al.*, 1997). HHV-8 ORF16 has been used studied for possible use in a diagnostic assay. An immunoblot assay, comprised of three HHV-8 recombinant proteins (ORF16, ORF57 and ORF71) was used to test the seroprevalence of HHV-8 in Taiwan (Wang *et al.*, 2002).

ORF65 encodes a protein which is homologous to the HHV-8 small viral capsid antigen (sVCA) ORF65, the EBV gene EFRF3 and to structural components of other herpesviruses including VP26 of herpes simplex virus (HSV), herpes virus saimiri (HVS) and a small capsid protein of human cytomegalovirus (CMV). These proteins are assumed to share related biological functions (Lin *et al.*, 1997) and it is likely that OvHV-2 ORF65 has the same function. They are capsid proteins and therefore likely to be expressed during the lytic cycle of viral replication. In EBV infections, antibodies to the viral capsid antigen persist, so that any antibody response to the capsid proteins is likely due to infection (Simpson *et al.*, 1996). There appears to be no antigenically cross-reactive response between HHV-8 ORF65 and the EBV gene EFRF3, despite homology (Lin *et al*, 1997). The HHV-8 ORF65 protein is an immunogenic lytic cycle-associated protein (Simpson *et al.*, 1996; Lin *et al.*, 1997). Herpesvirus virions share a characteristic architecture where the double-stranded DNA genome is surrounded by an icosahedral protein capsid, a tegument layer and a lipid bilayer envelope. The function of KSHV pORF65 is currently unknown, although its position on the outer regions of the capsid suggests that, it may regulate capsid-tegument interactions, in addition to possible DNA interactions.

As a means of identifying further potential antigens, the recent sequencing of the OvHV-2 genome provides the opportunity to identify genes of interest for the development of MCF-specific diagnostic reagents. It also allows the design of gene-specific PCR primers to amplify, clone, express and study isolated viral proteins. Seventy genes have been identified in the DNA sequence of OvHV-2, of which twelve are considered unique to MCF viruses and three are unique to the OvHV-2 genome (Hart *et al.*, 2007). These OvHV-2 unique genes are designated ORF Ov2.5, ORF Ov3.5 and ORF Ov8.5 (Hart *et al.*, 2007).

These genes were also selected as candidates for preliminary work due to their unique nature, since they were less likely to cross-react with other viruses. OvHV-2 ORF Ov2.5 encodes a 182-residue secreted protein, spliced from 5 exons,

with homology to IL-10. It encodes a functional IL-10 molecule which may block cytokine secretion by the proliferation of macrophages and mast cells (Stewart *et al.*, unpublished data). In contrast, the ORF Ov3.5 gene encodes a 163 residue secreted protein of unknown function, containing a putative signal peptide suggesting the encoded protein is secreted from infected cells. ORF Ov8.5 encodes a 390 residue protein with no similarity to any known protein (Hart *et al.*, 2007).

Additionally, polypeptides likely to be exported or expressed on the cell surface were selected as potential candidate antigens since they were more likely to be exposed to the immune system. ORF Ov7, a positional homologue of the EBV gene BZLF2, that encodes the glycoprotein g42 (Coulter *et al.*, 2001), was selected. The BZLF2 gene is involved in entry of virus into B cells via binding to HLA-DR (Sprigs *et al.*, 1996; Hart *et al.*, 2007), and is thought to be involved in receptor binding. Ov7 appears to encode the N-terminal part of this protein and may splice with the Ov8a and b exons to form the complete Ov7/Ov8 glycoprotein.

This chapter describes the bacterial expression of selected OvHV-2 genes. The expression of OvHV-2 genes ORF Ov8, Ov9 and ORF65 cloned in bacterial expression vectors was demonstrated using western blotting with epitope-tag-specific antibodies and anti-MCF antibodies in serum. Furthermore, genes Ov2.5, Ov3.5, Ov7 and Ov8.5 were selected as candidates due to their unique nature and were cloned and expressed to study the isolated viral proteins.

3.2 Materials and Methods

3.2.1 Amplification/ Cloning of OvHV-2 Unique Genes

3.2.1.1 Primer Design and SignalP V2.0.

Primers for OvHV-2 genes; Ov2.5, Ov3.5, Ov7 and Ov8.5 were designed with the program Primer 3 (Table 3.2) (URL: 1). OvHV-2 genes Ov8 Exon 1 2.1kb, Ov9 and ORF65 were previously cloned into expression vectors (Russell and Wiyono, unpublished data). Primers used to clone these genes are included in Table 3.2 for completeness. To create in-frame fusions when cloned into the expression vectors all of the gene fragments were given terminal NcoI sites in which the central ATG was in frame with the gene of interest.

OvHV-2 ORF	Start codon	Stop codon	Length (aa)	AlHV-1% identity (similarity)	Possible Function
Ov2.5	3081	3960	293	N/A	Il-10 homologue
Ov3.5	6416	6907	163	N/A	Unknown. Secreted?
Ov7	80406	80630	75	60	Glycoprotein
Ov8 2kb	81019	83209	-	-	Glycoprotein
Ov8 0.5kb	83209	83408	-	41	Glycoprotein
Ov8.5	117282	118454	390	N/A	Unknown
Ov9	124941	125561	206	50	Bcl2 homologue
ORF65	108568	107933	211	41	Capsid protein

Table 3.1: OvHV-2 gene fragments, coordinates, similarity to AlHV-1 and function. Gene start and stop positions are relative to OvHV-2 sequence coordinates. Two Ov8 exon 1 fragments were used, based on different predicted splice sites.

3.2.2. Expression Systems

Each OvHV-2 gene fragment was cloned into a TA vector (described in section 2.4.7) to check that PCR-amplified sequences were correct prior to transfer into expression vectors and transformation into the correct *E. coli* expression host. All cloned fragments had NcoI sites in-frame at both ends and the vectors used had C-terminal 6xHisTags for detection and purification. As this method was non-directional, cloned fragments were subject to restriction analysis to define insert orientation, using unique restriction sites in both the vector and insert. Restriction

enzymes (Promega) used include; Pst I (pTrxFus-ORF65, pET22b-Ov9, pET22b-ORF65, pBAD-Ov9 and pBAD-ORF65), Kpn I (pTrxFus-Ov8 and pBAD-Ov8) and Psp OMI (pET22b-Ov8).

Genes	Signal Peptide	Primer coordinates	Orientation	Sequence
Ov2.5	Yes	3163-3180	Forward	5'-CCATGGTCCTCCCCCTACGAGGTAA-3'
		3625-3651	Reverse	5'-CCATGGTGACCCCAAAGTAGCTTTCC-3'
Ov3.5	Yes	6483-6505	Forward	5'-CCATGGCCGCGGTAGACAGCTC-3'
		6882-6907	Reverse	5'-CCATGGCAGGTGGGGACTGTTGAGG-3'
Ov7	Yes	80498-80521	Forward	5'-CCATGGCCCTCTACAGGCAGCTA-3'
		80625-80649	Reverse	5'-CCATGGGGTAGCTGAACTCCACAC-3'
Ov8	No	81181-81200	Forward	5'-GGCGCGCCCATGGGGCCTGCGTACCCCAACTGA-3'
0.5kb		81651-81671	Reverse	5'GGCCGGCCCCATGGGCAGGTTGGTCAGTCGGTTAGG-3'
Ov8	No	81513-81527	Forward	5'-CTCTCCTGACGCGCCACCGTCCATGG-3'
2.1kb		83613-83593	Reverse	5'-TCAGCCCATGGATCCG-3'
Ov8.5	No	117282-11703	Forward	5'-CCATGGTGTCCCGGCTTCCT-3'
		118433-118456	Reverse	5'-CCATGGCAATAGACGGCGCCTGA-3'
Ov9	No	125435-125455	Forward	5'-CCATGGTGGAAGCTGGCATACT -3'
		126043-126025	Reverse	5'-CCATGGCGCGTGTGCATAACAGAAGCA-3'
ORF65	No	109065-109044	Forward	5'-CCATGGCGTACGCCAGGCCTCG-3'
		108447-108468	Reverse	5'-TCAGCCCATGGATCCCTTTTTGGGAGCTATGGGCT-3'

Table 3.2: OvHV-2 Primer sequences, primer orientation and signal peptide prediction by SignalP 3.0 server (CBS prediction server) program and URL: 2. Primer coordinates according to NCBI Blast, using the published sequence. Primer sequences for Ov8 (2.1kb), Ov9 and ORF65 were designed using an early annotation of the OvHV-2 sequence.

3.2.2.1. pBAD/His Expression System

Escherichia coli LMG194 cells were transformed with pBAD or pBAD with cloned OvHV-2 genes. The transformants were grown in LB containing 2 ml/l glycerol as the carbon source, supplemented with 50μg/ml-carbenicillin, in conical flasks at 37°C (225rpm). For expression, glycerol stock cultures of *E. coli* LMG194, carrying pBAD-Ov8 Exon 1 2.1kb, pBAD-Ov9 and pBAD-ORF65 were used to inoculate RM media (Appendix 1: 10ml containing 100μg/ml ampicillin). Cultures were incubated at 37°C (225rpm) until an OD_{600} of 0.5 was achieved. Cultures were induced with 0.2% L-arabinose and incubated at 37°C for optimum growth. Four hours after induction, the inoculated cultures were centrifuged at 3000 x g for 10 minutes. The supernatant was removed and the cell pellet stored at –20°C.

3.2.2.2. pET Expression System

Glycerol stock cultures of *E. coli* BL21 (DE3) carrying pET22b-Ov8 Exon 1 2.1kb, pET22b-Ov9 and pET22b-ORF65 were used to inoculate LB media (10ml containing 100μg/mg Ampicillin). Cultures were incubated at 37°C (225rpm) until an OD_{600} of 0.6-1 was achieved. Cultures were induced, with either IPTG (0.4mM) or the lactose-based Overnight Express™ Autoinduction System, and incubated at 37°C for optimum growth. Three hours after IPTG induction, the cultures were harvested at 3000 x g for 10 minutes. The supernatant was removed and the cell pellet stored at -20°C.

3.2.2.2.1. Overnight Express Autoinduction system (Novagen)

The Overnight Express™ Autoinduction Systems regulates protein expression in *E. coli* without addition of the synthetic inducer IPTG during growth. The overnight express autoinduction system is also intended to induce high-level protein production in IPTG-inducible bacterial expression systems without the need for monitoring of cell growth. Cell mass and target protein yield can be increased several fold as compared with conventional protocols using induction with IPTG (Novagen product user protocol TB363).

The Autoinduction system is comprised of three components: an induction solution (OnEx™ Solution 1) a buffering solution (OnEx Solution 2) and a magnesium solution (OnEx Solution 3). These three components allow regulated cell growth while maintaining culture pH, enabling high levels of target protein expression (Novagen user protocol, TB383 Rev. H. 1005).

Two hundred microlitres of the induction solution, 500μl of buffering solution and 10μl of magnesium solution were added to 9.29ml L-broth (glucose free), containing 100μg/ml ampicillin. The medium was inoculated with 10μl of glycerol stock and incubated at 37°C for 16 hours, shaking at 225 rpm. Following incubation, the cultures were centrifuged at 3000 x g for 10 minutes, and the cell pellet stored at -20°C.

3.2.2.3. ThioFusion™ Expression System

The target genes, once cloned into the pTrxFus plasmid, were transformed into the thioredoxin expression host (GI724) to allow regulated expression of the fusion protein. Glycerol stock cultures of *E. coli* G1724 carrying pTrxFus-Ov8 Exon

1 2.1kb and ORF65 were inoculated onto RM Medium agar plates with ampicillin (100μg/ml) and incubated overnight at 30°C. Single colonies were used to inoculate 100ml induction medium (RM media with 20g casamino acids/l) containing 100μg/mg ampicillin and the cultures were incubated at 30°C (225rpm) until an OD_{550} of 0.5 was achieved. Cultures were induced with 100μg/ml tryptophan and incubated at 37°C for optimum growth. Four hours after induction, the cultures were harvested by centrifugation at 3000 x g for 10 minutes. The supernatant was removed and the cell pellets stored at -20°C.

3.2.3. Expression Analysis

3.2.3.1. Preparation of cleared lysates for protein purification

In order to determine whether the expressed proteins were soluble, crude extracts were made by mechanical lysis of bacterial cultures using an Eaton Press pressure chamber. Cells were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole. pH 8.0) and placed into a chilled (-70°C) pressure cell. The frozen pellet liquefies under pressure and passes from the upper chamber to the lower chamber of the cell, the resulting rapid drop in chamber pressure causing cell lysis. The cell lysates were collected and the insoluble debris pelleted by centrifugation for 30 minutes at 11,250 x g. Sample aliquots were stored at -20°C.

3.2.3.2. Protein Miniprep of 6×His-Tagged proteins from *E. coli* under native conditions

Small-scale purifications of His-tagged fusion proteins were carried out on 1ml of soluble protein lysates in order to test proteins present in the expressing cells. This procedure was carried out in accordance with the manufacturer's instructions (The QIAexpressionist 08/2002, Qiagen).

The cell lysate supernatants were centrifuged for 1 minute at 12000 x g to remove any cellular debris and were transferred to a fresh 1.5ml microcentrifuge tube. 20μl of a 50% slurry of Ni-NTA resin (Ni-NTA Agarose, Qiagen) was added to 1ml cell lysate and mixed gently for 30 minutes at 4°C. The mixture was then centrifuged for 10 seconds at 1000 x g to pellet the resin and the supernatant was removed and kept. The pelleted resin was washed twice in 100μl of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole. pH 8.0) to remove unbound and weakly interacting proteins. The supernatant from each wash was stored at -20°C.

Bound proteins were then eluted by washing three times in 20µl elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole. pH 8.0). The resulting supernatants were removed and combined. Both the remaining Ni-NTA slurry pellet and all supernatants were stored at -20°C.

3.2.3.3. Gel Filtration – Superdex200

As an alternative to purification using the Ni-NTA resin, gel filtration was used to fractionate lysates according to size (described in section 2.7.2).

3.3. Results

3.3.1. Bacterial systems

Three different expression vectors were used for the expression of OvHV-2 recombinant proteins, each encoding a 6-histidine tag to facilitate protein purification: (i) pBAD/His (Invitrogen), (ii) pET-22b (Novagen) and (iii) pTrxFus (Invitrogen). Results of bacterial expression systems tested are summarised in table 3.3, and described in detail in section 3.3.2-3.3.5.

Cloning of the OvHV-2 genes was accomplished by using OvHV-2 genomic DNA as the template for PCR-based amplification with appropriate oligonucleotide primers carrying terminal NcoI sites. The PCR products were cloned into NcoI sites of each selected vector. Of all of the gene/vector combinations selected, only the Ov9-pTrxFus cloning was unsuccessful, despite several attempts (Russell & Wiyono, unpublished finding). Analysis of several clones from these attempts showed a significant level of rearrangement when Ov9 was cloned into pTrxFus, therefore pTrxFus-Ov9 was considered unviable.

OHV-2 gene	Possible functions	Bacterial Expression	Soluble (S) or Insoluble (I)	Purified
Ov2.5	**IL-10 homologue**	pT	I	ND
Ov3.5	**Unknown. Secreted?**	×	×	ND
Ov7	**Glycoprotein**	pT	I	ND
Ov8 (2.1kb)	**Glycoprotein**	pB, pE, pT	I	×
Ov8 (0.5kb)	**Glycoprotein**	pT	S	×
Ov8.5	**Unknown**	×	×	×
Ov9	**bcl2 family**	×	×	ND
ORF65	**Capsid protein**	pB, pE, pT	S	Enriched

Table: 3.3: Summary of OvHV-2 gene functions, bacterial expression systems tested, solubility and purification. pB represents the pBAD expression system, pE represents the pET expression system (pET-22b) and pT represents Thioredoxin expression system (pTrxFus). × indicates unsuccessful expression or purification, ND – not done.

3.3.2. pBAD Expression System:

3.3.2.1. OvHV-2 Ov8 Exon 1 2.1kb, Ov9 and ORF65 in the pBAD/His Vector

OvHV-2 clones in the pBAD expression vector were analysed in *E. coli* strain LMG194. Cultures of each clone, together with the empty pBAD vector, were grown and induced by the addition of L-arabinose. Cells were harvested four hours after induction. Samples of induced cultures were lysed in 2x SDS loading buffer and 10μl of diluted supernatant (1:2) from each lysate was electrophoresed on a 10% NuPAGE tris-glycine polyacrylamide gel. The gel was stained with Coomassie blue stain. No obvious bands for pBAD-Ov8 Exon 1 2.1kb, pBAD-Ov9 or pBAD-ORF65 were distinguished, indicating no protein over-expression (Figure 3.8a).

A western blot was performed using an anti-HisTag monoclonal antibody, to determine whether any recombinant protein was present (Figure 3.8b). The pBAD vector alone expressed a single polypeptide at approximately 16kDa representing a 45 residue polypeptide encoded by the empty vector. pBAD-Ov8 Exon 1 2.1kb expressed two bands at approximately 80kDa and 100kDa (expected size 80kDa), while pBAD-ORF65 expressed a single band at approximately 25kDa, with an expected size of 28kDa. No expression was observed for pBAD-Ov9, which has an expected size of 25kDa (Figure 3.8b).

3.3.2.2. pET Expression System:

3.3.2.2.1. OvHV-2 Ov8 Exon 1 2.1kb, Ov9 & ORF65 in the pET-22b Vector

OvHV-2 clones in the pET-22b expression vector were analysed in *E. coli* strain BL21 (DE3). Ten millilitre cultures of each clone, together with the empty pET22b vector, were grown in LB media, and induced by IPTG (0.4mM), while replicate cultures were induced by the Overnight Express system (Novagen). Samples of induced cultures were lysed in SDS loading buffer at equivalent cell densities and 10μl of each crude extract were electrophoresed on 10% NuPAGE tris-glycine gels. The gels were stained with Coomassie blue stain or transferred to nitrocellulose for antibody staining (Figure 3.9.). No obvious pET22b-Ov8 Exon 1 2.1kb (80kDa), pET22b-Ov9 (25kDa) or pET22b-ORF65 (28kDa) bands were distinguished, indicating no obvious protein over-expression.

To confirm the presence of pET22b-Ov8 Exon 1 2.1kb, pET22b-Ov9 and pET22b-ORF65 His-tagged proteins in the lysates, a western blot using anti-HisTag antibody was performed (Figure 3.9). Both induction methods showed a number of

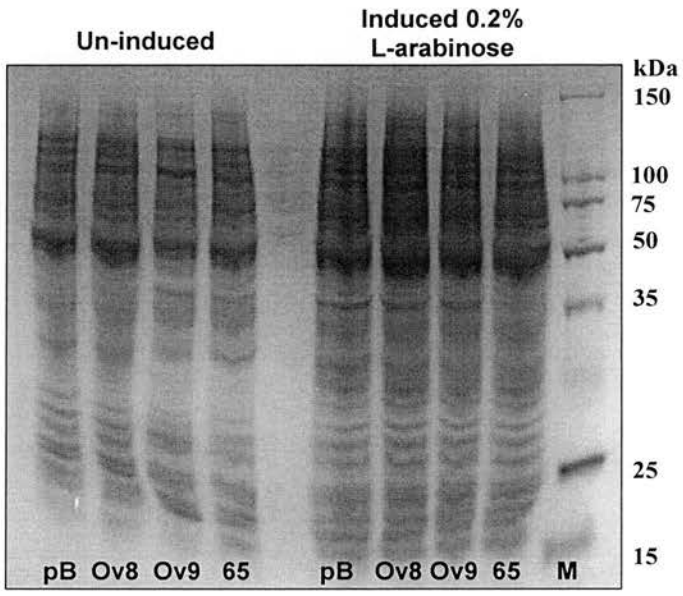


Figure 3.8a: Electrophoretic analysis of pBAD expression. Total cell lysates (10μl) of bacteria containing plasmids pBAD-Ov8, pBAD-Ov9, pBAD-ORF65 were separated by electrophoresis on a 10% NuPAGE Novex tris-glycine gel and stained with Coomassie blue. Bacteria containing the empty pBAD vector (pB) were present as a control. Un-induced and Induced (>4 hours after induction with 0.2% L-arabinose) were compared.

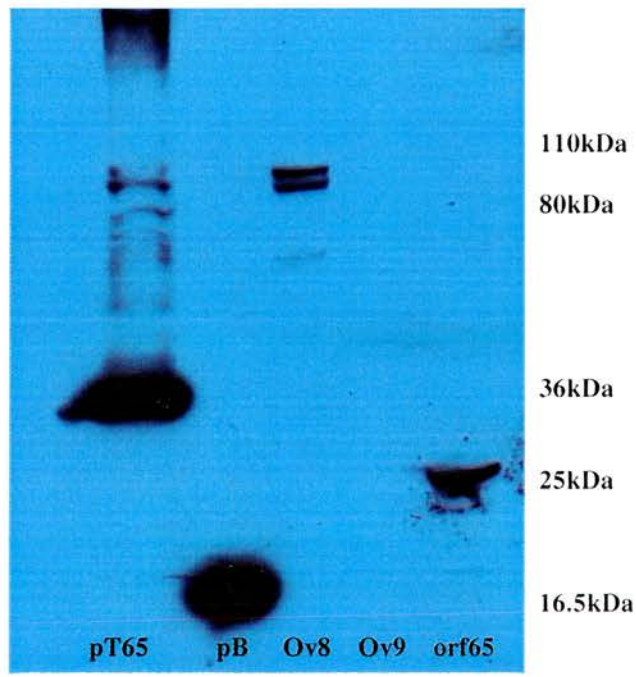


Figure 3.8b: Chemiluminescent analysis of pBAD expression system.

Whole bacterial lysates (20µl) containing pBAD plasmids pBAD-Ov8, pBAD-Ov9 and pBAD-ORF65 were separated by electrophoresis, on a 10% NuPAGE Novex tris-glycine gel, together with the pBAD empty vector (pB) and pTrxFus-orf65 (pT65), present as controls. The lysates were analysed by western blotting using anti-HisTag antibody as a primary antibody followed by anti-mouse HRP conjugate. The bound conjugate was detected by chemiluminescence using the Pierce Supersignal ELISA femto HRP substrate.

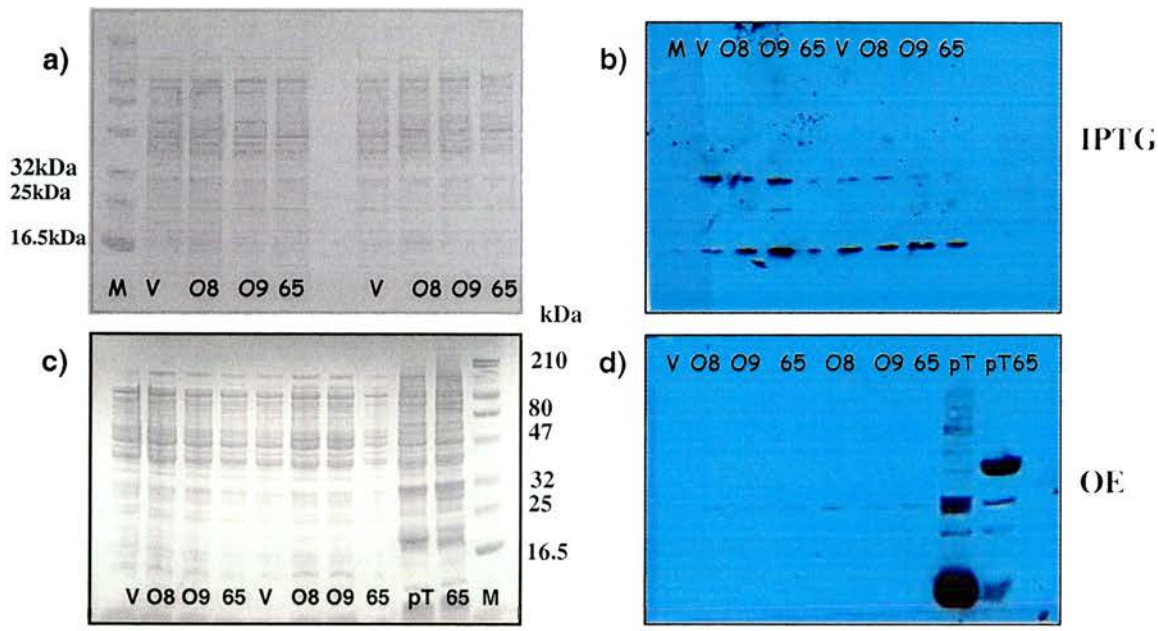


Figure 3.9: Electrophoretic analysis of total cell lysates from pET system.

Ten microlitres (duplicate) of whole bacterial lysate for pET22b-Ov8, pET22b-Ov9, pET22b-ORF65 and the pET-22b vector (v), induced by either IPTG (a and b) or the Overnight Express (OE) Autoinduction System (c and d). Proteins were separated on a 10% NuPAGE Novex tris-glycine gel and were either stained with Coomassie blue (a & c) or analysed by western blot (b & d) using an anti-HisTag monoclonal antibody and visualised with chemiluminescence using the Pierce Supersignal ELISA femto HRP substrate.

non-specific bands but no OvHV-2 protein bands were seen. The His-tagged thioredoxin control and pTrxFus-ORF65 were detected on the gel containing the overnight express system-induced cultures suggesting induction of pTrxFus was more efficient by over night expression.

Repeating the standard IPTG induction method, a western blot of the gene products was carried out. pET22b-Ov9 and pET22b-ORF65 gene products showed no His-Tagged bands, while the pET22b-Ov8 Exon 1 2.1kb crude lysate showed two bands, of approximately 80kDa and 100kDa in size (Figure 3.10). A possible explanation for the two bands may simply be that the pET22b-Ov8 Exon 1 2.1kb protein has been cleaved into two stable products, with and without the vector-encoded leader peptide sequence (21aa). However, the size difference of 30kDa suggests a larger fragment may be cleaved off the Ov8 fragment. The Ov8 gene was also cleaved in the pBAD system, implying this occurs within the Ov8 sequence. In the pET-22b expression system only the OvHV-2 cloned Ov8 fragment expressed a band of the expected size that could be detected by western blotting (3.10).

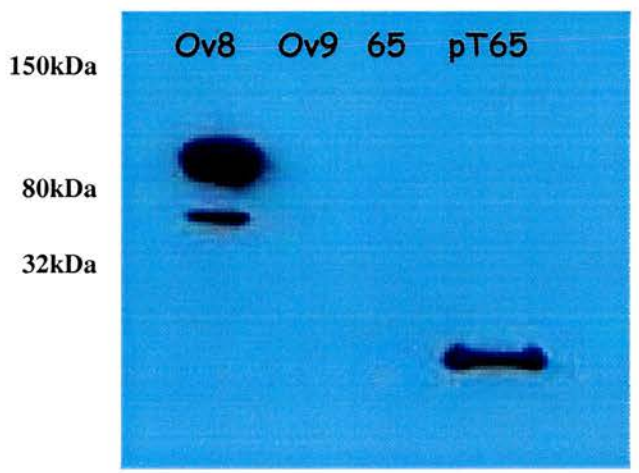


Figure 3.10: IPTG induced gene products. Total bacterial cell lysates from bacteria containing pET22b plasmids pET22b-Ov8, pET22b-Ov9, pET22b-ORF65 were separated by electrophoresis on a 10% NuPAGE Novex tris-glycine gel, and analysed by western blot using an anti-HisTag monoclonal antibody and visualised with chemiluminescence using the Pierce Supersignal ELISA femto HRP substrate. Bands present were compared to a protein marker (15-150kDa). pTrxFus-ORF65 was used as a positive control.

3.3.2.2.2. Batch purification of pET-22b Ov8

Since initial pET-22b-Ov8 Exon 1 2.1kb extracts showed an expressed His-tagged protein, a second batch of pET22b-Ov8 Exon 1 2.1kb culture (50ml) was grown to analyse the presence of expressed pET22b-Ov8 protein. The cells were mechanically lysed and a western blot was performed using anti-HisTag monoclonal antibody (Figure 3.11).

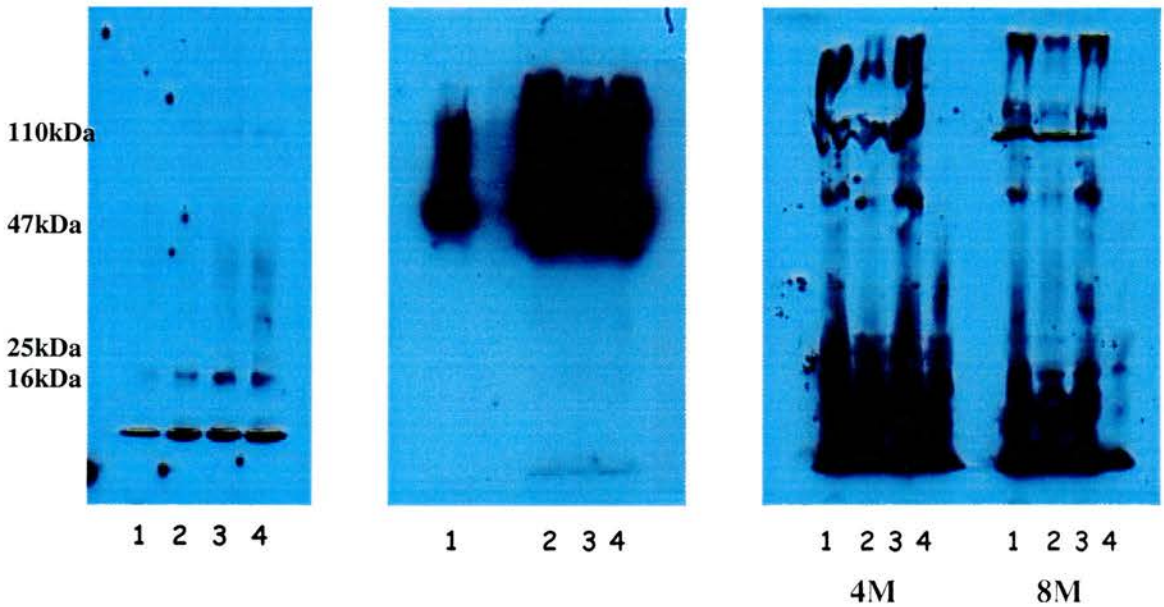


Figure 3.11a, b & c: Chemiluminescent western blot to determine solubility of pET22b-Ov8 gene product. Ten microlitres of bacterial lysate containing pET22b-Ov8 a) whole cell crude lysate and b) cell pellet was separated by electrophoresis on a 10% NuPAGE Novex tris-glycine gel, were analysed by western blotting, using anti-HisTag monoclonal antibody as a primary conjugate. c) To aid solubility, 50µl of the insoluble gene product was treated with two concentrations of urea (4M and 8M). The numbers given to each samples represent different OvHV-2-pET22b-Ov8 clones. The blot was visualised with chemiluminescence using the Pierce Supersignal ELISA femto HRP substrate and any bands compared to a 15-150kDa protein marker.

The pET22b-Ov8 Exon 1 2.1kb soluble lysate showed some background but no over-expressed protein in the expected position (Figure 3.11a). The cell pellet had a signal near the expected position (Figure 3.11b) but no clear bands were seen. This suggested that the pET22b-Ov8 Exon 1 2.1kb protein was insoluble and reduced poorly on SDS-PAGE polyacrylamide gels. Concentrated urea solution (8M) was used to dissolve the cell pellet. 20µl aliquots of the urea-treated samples were then electrophoresed on a 10% NuPAGE tris-glycine gel and a western blot was

performed, using anti-HisTag antibody (Figure 3.11c). In this case high background was seen in the low molecular weight part of the gel but no clear over-expressed pET22b-Ov8 Exon 1 2.1kb bands could be distinguished. The proteins may have solubilised to a certain extent in the presence of concentrated urea, although most of the signal in the soluble urea material seemed to be due to low molecular weight proteins or fragments. It is therefore possible that the pET22b-Ov8 Exon 1 2.1kb product was essentially insoluble. Alternatively, the western blot may not have worked properly, as indicated by the high background when compared by western blots such as figure 3.11a.

3.3.2.3. ThioFusion Expression System

3.3.2.3.1. OvHV-2 ORF Ov8 Exon 1 2.1kb & ORF65 in the pTrxFus Vector

pTrxFus carrying the OvHV-2 Ov8 Exon 1: 2.1kb and ORF65 genes in *E. coli* GI724 and the empty pTrxFus vector, were grown in 10ml cultures, in RM media until an OD_{550} of 0.5 was reached. The cultures were induced by the addition of 100µg/ml tryptophan. Cells were harvested four hours after induction. Bacterial lysates were prepared in SDS loading buffer and 10µl of each lysate was electrophoresed on a 10% NuPAGE bis-tris polyacrylamide gel. The gel was stained with Coomassie blue stain (Figure 3.12a). No obvious bands at the expected size for either pTrxFus-Ov8 Exon 1 2.1kb or pTrxFus-ORF65 were seen.

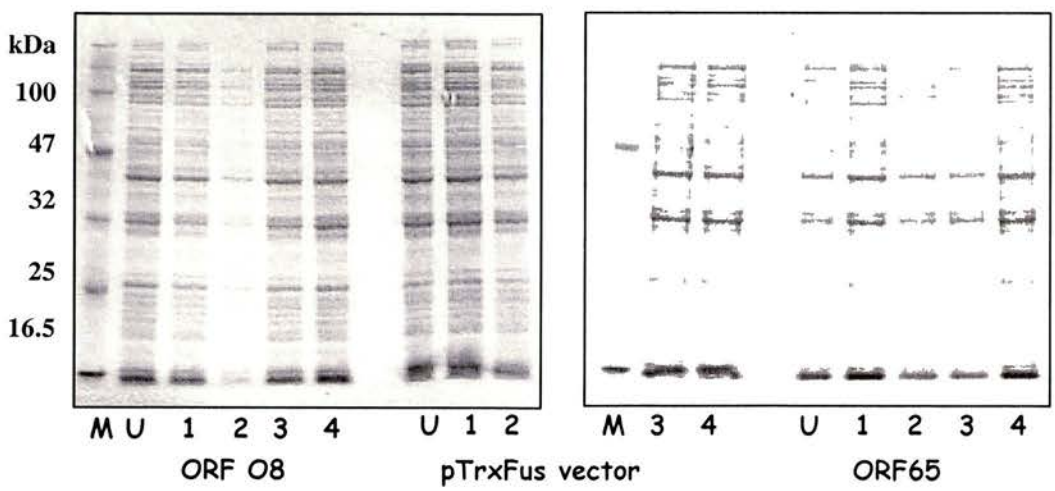


Figure 3.12a: Coomassie blue stained gel of total cells lysate extracts from Thiofusion expression system cultures. Samples of bacterial cultures containing the vector, pTrxFus-Ov8 and pTrxFus-ORF65 were taken at hourly intervals after induction with Tryptophan (1-4 hours) were electrophoresed on a 10% NuPAGE Novex bis-tris gels. M represents the protein marker. U represents uninduced samples.

To determine whether the pTrxFus, pTrxFus-Ov8 Exon 1 2.1kb and pTrxFus-ORF65 protein were found in the soluble phase, the induced cultures (4 hours induction with L-arabinose) were mechanically lysed and the soluble fraction was analysed on a western blot, using an anti-HisTag monoclonal antibody (Figure 3.12b). The pTrxFus vector and pTrxFus-Ov8 Exon 1 2.1kb expressed a single His-tagged band at approximately 16.5kDa, the size expected for unfused thioredoxin, while pTrxFus-ORF65 expressed a single band at approximately 32kDa, the size expected for the ORF65 fusion. So pTrxFus and pTrxFus-ORF65 expressed a soluble band of the expected size, while pTrxFus-Ov8 Exon 1 2.1kb showed a band of the same size as thioredoxin. One explanation for the inconsistency regarding the pTrxFus-Ov8 Exon 1 2.1kb fusion protein could be that the Ov8 clones do not have the correct insert, or that the insert has been deleted. Alternatively, it may suggest a problem with the expression of the pTrxFus-Ov8 Exon 1 2.1kb gene product or proteolysis of the fusion protein.

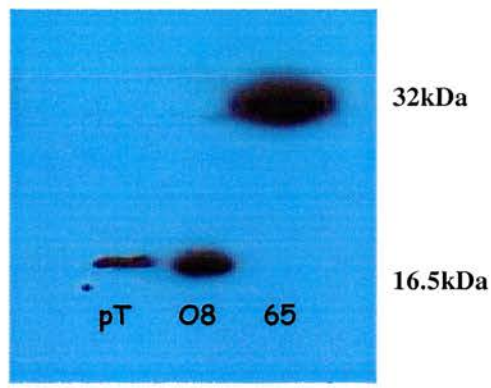


Figure 3.12b: Western blot of Thiofusion expression system crude extracts. Twenty micrograms of crude lysate pTrxFus-Ov8 and pTrxFus-ORF65 (> 4 hours after induction with Tryptophan) were electrophoresed alongside the pTrxFus empty vector (pT), present as a control. The electrophoresed protein lysate was analysed by western blotting using anti-HisTag antibody as a primary antibody followed by anti-mouse HRP conjugate. The bound conjugate was detected by chemiluminescence using the Pierce Supersignal ELISA femto HRP substrate.

3.3.2.3.2. Ni-Resin Purification

To isolate target pTrxFus-Ov8 Exon 1 2.1kb and pTrxFus-ORF65 His-tagged proteins, affinity purification was performed. Purification of the pTrxFus-Ov8 Exon 1 2.1kb and pTrxFus-ORF65 his-tagged soluble lysate was attempted using a nickel

affinity resin purification technique. Ten microlitres of each wash and eluent, along with the resin-bound residue, were subjected to electrophoresis on a 10% NuPAGE bis-tris gel. The gel was stained overnight with Coomassie blue stain (Figures 3.13).

The soluble crude lysates of each sample were heavily loaded but no over-expressed bands could be distinguished (Figure 3.13), suggesting all proteins are bound to the column. Although the lysate has undergone a ten-fold dilution, the bands in the crude extract are only faintly visible in the unbound supernatant, suggesting most proteins in the lysates bound to the resin.

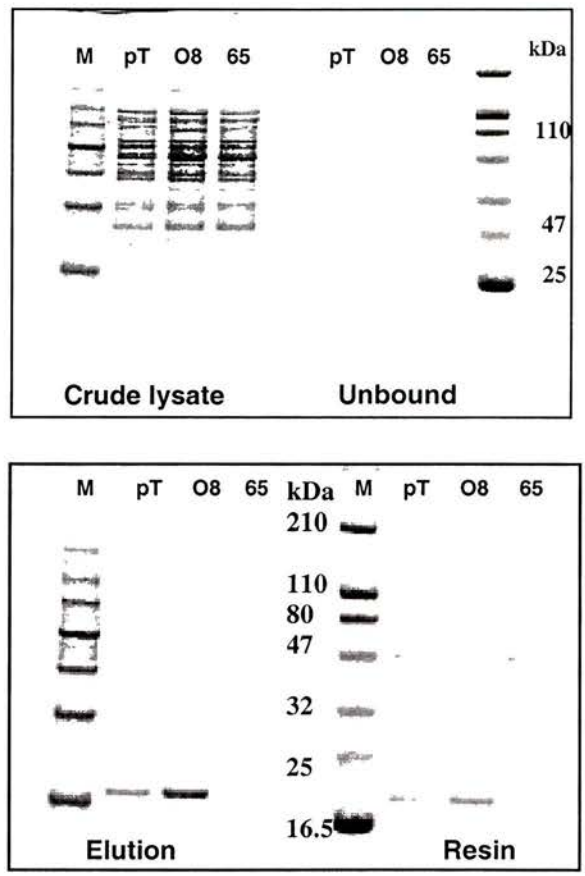


Figure 3.13: Ni-NTA resin purification of pTxfus fusion proteins Ov8 and ORF65. Purification of pTrxFus fusion proteins. Extracts from pTrxFus-Ov8 (O8) and pTrxFus-ORF65 (65), electrophoresed on a 10% NuPAGE® Novex bis-tris gels and stained with Coomassie blue stain. The crude lysate shows a high level of protein expression, while wash fractions and elution stages of the purification show a low level of protein expression suggets the majority of protein remained bound to the Ni-NTA resin. The empty pTrxFus vector (pT) was used as a control.

An enriched protein band at 16.5kDa was clearly visible in the pTrxFus and pTrxFus-Ov8 Exon 1 2.1kb eluent, while no enriched/purified band for pTrxFus-ORF65 was obvious (Figure 3.13). pTrxFus-Ov8 shows unfused thioredoxin. The resin-bound residue for pTrxFus and pTrxFus-Ov8 Exon 1 2.1kb showed non-specific background with an enriched protein band at 16.5kDa (Figure 3.13). The presence of thioredoxin on the agarose beads was probably due to the high level of expression and to difficulties eluting bound protein, using a simple batch method. pTrxFus-ORF65 was not seen on the resin, presumably because there was possibly either less bound protein or that it eluted better. Therefore while there was enrichment of unfused thioredoxin derived bands in the eluent, there was no obvious band representing pTrxFus-ORF65. Although this result may reflect a lower expression level of the pTrxFus-ORF65 fusion in this system, expression of this fusion protein in soluble form was clearly shown in the crude lysate (Figure 3.12b). This result suggests there may have been a problem with the purification of the protein by affinity purification methods, resulting in a low level of binding in the eluted fraction, after being exposed to the nickel resin. A possible explanation for this lack of protein binding may be a result of protein remaining bound to the chelating metal resin agarose beads.

To confirm the presence of the His-tagged proteins, a western blot, using anti-HisTag antibody, was performed (Figure 3.14a, b) on samples from the purification procedure. A distinct band was observed in each of the crude lysate samples analysed, indicating all samples contained expressed, His-tagged protein (as seen in figure 3.12). pTrxFus and pTrxFus-Ov8 Exon 1 2.1kb wash supernatant (unbound) showed a single 16.5kDa band, suggesting a proportion of these proteins had not bound to the resin. No bands were observed in the pTrxFus-ORF65 wash (Figure 3.14a). This probably reflected the relative expression level of each protein and the ability of the resin to bind all of the His-tagged protein from each extract. Eluted pTrxFus and pTrxFus-Ov8 Exon 1 2.1kb samples showed strong bands at 16.5kDa, representing enriched proteins, and this corresponded to the results of the Coomassie stained gel (Figure 3.13). Additionally, pTrxFus-ORF65 was seen at 32kDa, with a weak band visible at 25kDa. This was clearly enrichment, but not a clean purification since no distinct band of 32kDa was seen in figure 3.13.

Furthermore, the 25kDa band in the pTrxFus-orf65 eluent suggests that some of the bound protein may have been degraded. The resin-bound residues of pTrxFus and pTrxFus-Ov8 Exon 1 2.1kb showed HisTag positive bands at 16.5kDa, while the

pTrxFus-ORF65 resin showed a strong band at 32kDa, suggesting that in all 3 cases some of the bound protein was not eluted from the Ni-NTA resin.

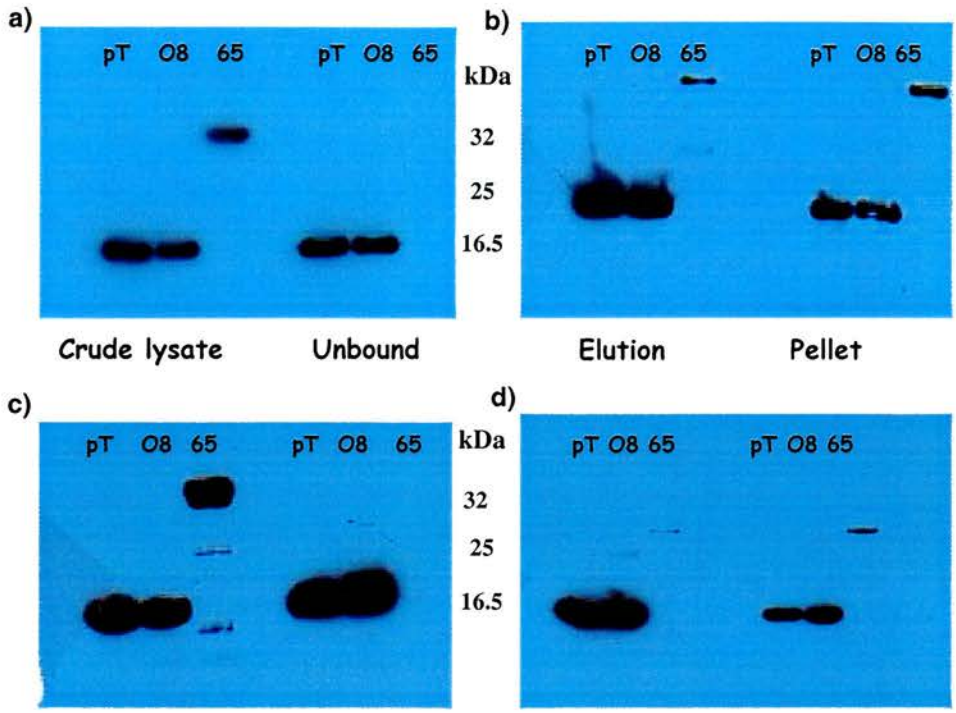


Figure 3.14: Chemiluminescent western blot of Ni-NTA purification.
pTrxFus-Ov8 and pTrxFus-ORF65, electrophoresed on a 10% NuPAGE Novex bis-tris gel, were analysed by western blotting using HisTag antibodies (a & b) and Anti-Thio antibodies (c & d). The pTrxFus empty vector (pT) was present as a control. Blots were visualised with chemiluminescence using the Pierce Supersignal ELISA femto HRP substrate.

A western blot was also performed using anti-Thio antibody. The Anti-Thio antibody detects thioredoxin (Figure 3.14c, d), binding to the N-terminal end of the fusion proteins. This antibody was used to test whether the thioredoxin domain was present on each fusion or degraded. As before, the same banding pattern was observed with the exception that three bands (32kDa, 25kDa and 16.5kDa) were observed in the pTrxFus-ORF65 crude extract sample further confirming that the pTrxFus-ORF65 fusion was sensitive to degradation and that the thioredoxin fragment of the fusion appeared to be one of the degradation products. Thus, both the anti-HisTag antibody and the anti-Thio antibody show similar sized bands at 32kDa and 25kDa, while the anti-Thio antibody showed an extra band at around 16kDa suggesting degradation of the His-Tagged thioredoxin-orf65 protein. The HisTag is

located on the C-terminus of the protein, while the anti-Thio epitope is located at the N-terminus. The bands at 32 and 25kDa are likely a result of N-terminal degradation since the HisTag is still present. However, cleavage to release the anti-Thio epitope may also occur, as the 16kDa band is only deleted by the anti-Thio antibody.

Furthermore, a weak pTrxFus-Ov8 Exon 1 2.1kb band was visible at 25kDa in the wash and eluent using anti-Thio antibody, which was not seen in the His-Tag blot. This may indicate proteolysis of the fusion protein within the Ov8 segment. The lack of a band at the expected size from the pTrxFus-Ov8 Exon 1 2.1kb construct (80kDa) again suggests that the Ov8 clones prepared have an incorrect insert or indicate a problem during expression. Therefore, the structure of the pTrxFus-Ov8 Exon 1 2.1kb from constructs was checked by digestion with restriction enzymes.

3.3.2.3.3. Analysis of pTrxFus Ov8 Exon 1 2.1kb construct

Analysis of the Ov8 Exon 1 2.1kb clones within the three expression systems highlighted a problem with the Ov8 insert. The pBAD and pET expression systems show two bands at 80 and 100kDa, rather than the single band expected. Possible explanations for the expression of two bands may indicate the Ov8 has been cleaved. As this cleavage was shown in both expression systems it indicates the Ov8 sequence rather than the expression vectors is responsible. Further demonstration of an inconsistency within the Ov8 Exon 1 2.1kb sequence was shown using the thioredoxin expression system, as only a band representing the empty thioredoxin vector was observed. This result confirmed an irregularity within the Ov8 clones and suggests possible deletion of the insert. Therefore, to check that the pTrxFus-Ov8 Exon 1 2.1kb construct contained an insert of the correct size, pTrxFus-Ov8 Exon 1 2.1kb samples from four independent glycerol stocks in E. *coli* expression strain GI724 (pTrxFus) were used to prepare plasmid DNA for restriction enzyme analysis. The plasmid DNA was digested with NcoI, which was used to insert the Ov8 fragment, and electrophoresed on a 0.8% agarose gel (Figure 3.15).

The pTrxFus vector showed a single band of 4kb. The four independent pTrxFus-Ov8 Exon 1 2.1kb isolates all showed the same two bands, a 4kb vector

band and a 2kb band corresponding to the Ov8 insert (2085bp). For comparison, pTrxFus-ORF65 showed a 4kb vector band and a 600bp band corresponding to the ORF65 insert. Thus all of the constructs used, and all of the pTrxFus-Ov8 Exon 1 2.1kb isolates tested, had the expected structure. This did not explain the discrepancy between the observed size of the anti-Thio and anti-HisTag reactive band and the predicted size of the Ov8 thioredoxin fusion as the DNA appears intact.

A possible explanation for this discrepancy may simply be that the pTrxFus-Ov8 Exon 1 2.1kb protein is rapidly degraded into two stable products of similar size, one containing the N-terminal thioredoxin epitope and the other containing the C-terminal HisTag epitope. This is supported by the detection of a small amount of a 24kDa band in the elution sample of pTrxFus-Ov8 Exon 1 2.1kb which is evidence of degradation (Figure 3.14d).

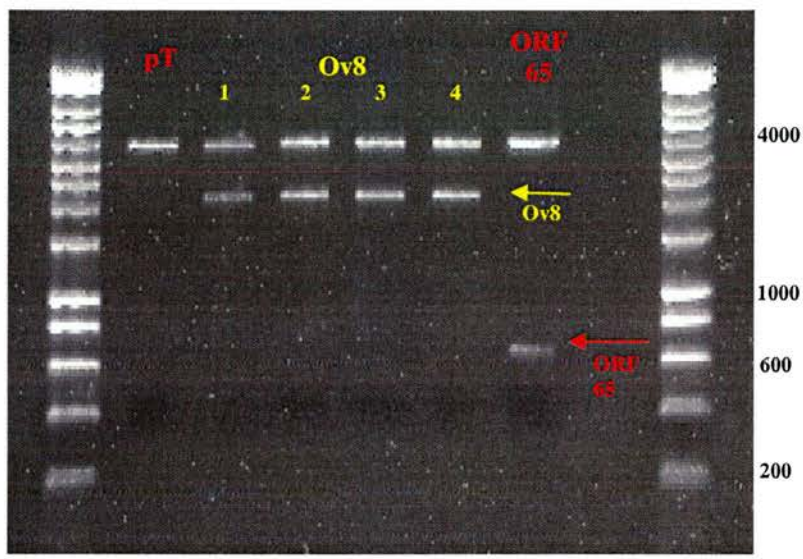


Figure 3.15: Analysis of thioredoxin protein fusions.

DNA was prepared from glycerol stocks using a QIAGEN miniprep kit. DNA from pTrxFus-Ov8 and pTrxFus-ORF65 gene products were digested with restriction endonuclease enzyme NcoI and electrophoresed on a 0.8% agarose gel alongside a 1kb DNA ladder. The empty pTrxFus (pT) vector was present as a control. The numbers given to each DNA sample represent different glycerol stocks.

3.3.2.3.4. Batch purification of pTrxFus ORF65

Since the initial purification of pTrxFus-ORF65 showed possible degradation, a second batch of pTrxFus-ORF65 culture was mechanically lysed in the presence of Complete (TM) EDTA-free protease inhibitors (Roche) to minimise degradation.

pTrxFus-Ov8 Exon 1 2.1kb was not tested under these conditions due to insolubility. When a cell is disrupted, proteases are released that can degrade any proteins. This degradation results in a reduced protein yield during isolation and purification. EDTA-free protease inhibitors were used as they do not affect the stability or function of metal-dependent proteins (i.e. the HisTagged proteins).

A western blot (Figure 3.16), using anti-HisTag monoclonal antibody, was employed to analyse the presence of pTrxFus-ORF65 protein in the pellet and supernatant of the crude lysate. The pTrxFus cell pellet contained a high level of insoluble thioredoxin protein with a main band of approximately 16.5kDa. This may reflect solubility of His-Tagged thioredoxin, or incomplete cell lysis. The soluble lysate showed two bands of approximately 16.5kDa and 30kDa. This may indicate aggregation of thioredoxin in the presence of protease inhibitor or the effect of EDTA. Interestingly, the pTrxFus-ORF65 cell pellet also showed two bands of approximately 25kDa and 32kDa in size, while the soluble lysate showed a single band at the expected size of 32kDa. The absence of the second band may indicate that the degraded protein fragments remained insoluble. These results suggest that the pTrxFus-ORF65 thioredoxin fusion is found mainly in the pellet material and is subject to degradation.

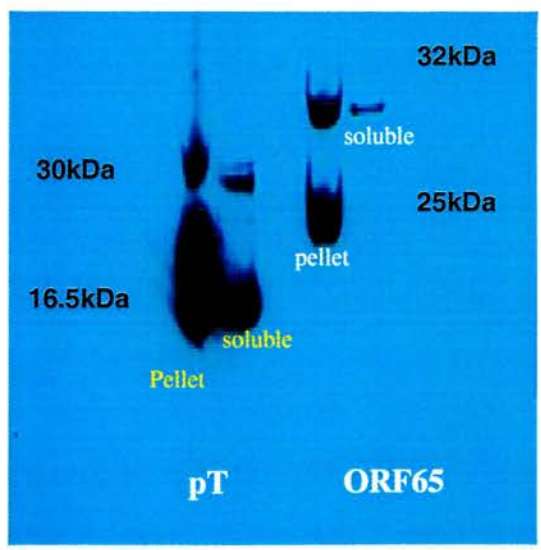


Figure 3.16: Chemiluminescent western blot of pTrxFus-ORF65 soluble and insoluble fractions. Ten microlitres of soluble and insoluble pTrxFus-ORF65 bacterial extract was electrophoresed alongside samples from pTrxFus extracts, present as a control (pT). The electrophoresed protein was analysed by western blotting using anti-HisTag primary antibody and visualised with chemiluminescence using the Pierce Supersignal ELISA femto HRP substrate.

Larger scale crude lysates of His-tagged pTrxFus and pTrxFus-ORF65 were purified using the nickel resin batch purification technique. The crude lysate, wash, eluant and resin-bound residue were run on a 10% tris-glycine gel and stained with Coomassie blue stain (Figure 3.17).

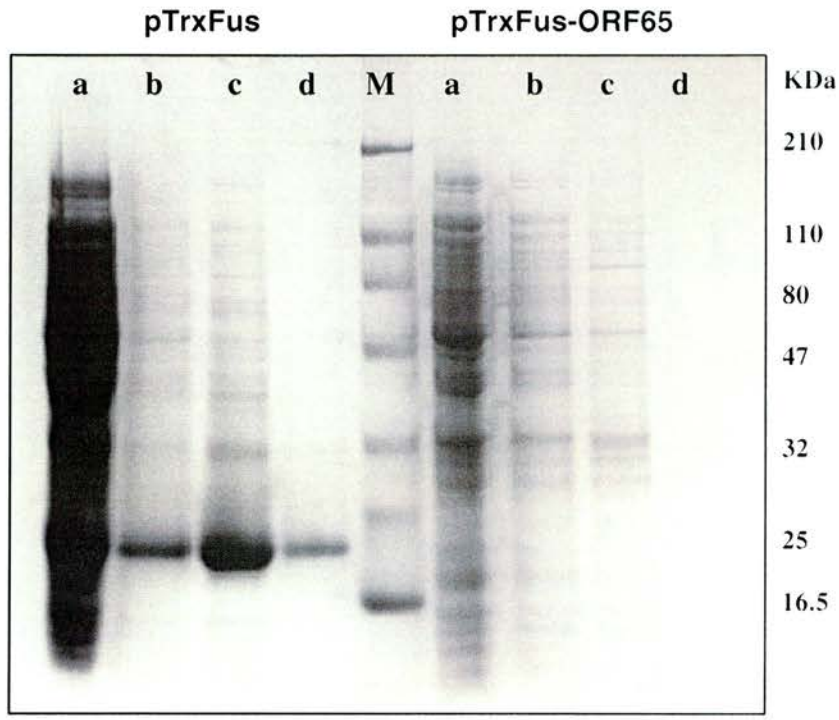


Figure 3.17a: pTrxFus ORF65 Ni-NTA purification. Ni-NTA resin purified pTrxFus-ORF65 and pTrxFus samples (10µl) were electrophoresed on a 10% NuPAGE® Novex bis-tris gel and stained overnight in Coomassie blue stain. The gel shows the four stages of Ni-NTA resin; (a) represents the untreated crude lysate (b) represents samples in the wash fraction, while (c) and (d) represents the first and third elution steps.

The pTrxFus crude lysate was heavily loaded, so no over-expressed band could be distinguished. However, thioredoxin (16.5kDa) was visible in the wash fraction suggesting it was highly over-expressed in this experiment and did not all bind to the resin or was not tightly bound and washed off. This band was also seen in the eluents, especially in the second eluent (Figure 3.17a) lane d where it is the only band visible. These results corresponded with those in the western blot (Figure 3.17b). In contrast, there was no obvious purification of the pTrxFus-ORF65 fusion protein on the stained gel (Figure 3.17a) with the wash and elution lanes not looking significantly different (Figure 3.17a). The western blot showed two visible bands at

25kDa and 32kDa in the pTrxFus-ORF65 crude lysate and wash-supernatant. The wash-supernatant was 10 times more dilute than the lysate, with only 10% of the overall fraction being used, while the eluent was 500 times more dilute than the lysate, with 50% of the total being loaded onto the gel. It is clear that for ORF65 the elution contains less total protein than the wash, but clearly has more His-tag signal on the blot. pTrxFus-ORF65 appears to have been enriched. Interestingly, no bands were visible in lane four, the final eluent, although two strong bands were visible in the previous eluent suggesting all of the protein had eluted early. Thus while pTrxFus appeared to be significantly purified by this method, pTrxFus-ORF65 was only enriched by Ni-NTA but not purified. This may reflect a lack of stringency in the wash procedure employed or the availability of the HisTag for binding to the resin in this fusion protein, in addition to the poor expression of the pTrxFus-ORF65 fusion. It is also clear (Figure 3.17a) that the ORF65-thioredoxin fusion was less well expressed than thioredoxin alone, and that other proteins bind to the resin and were seen in the eluant. With thioredoxin, all other Ni-NTA binding proteins appeared to be competed off the column.

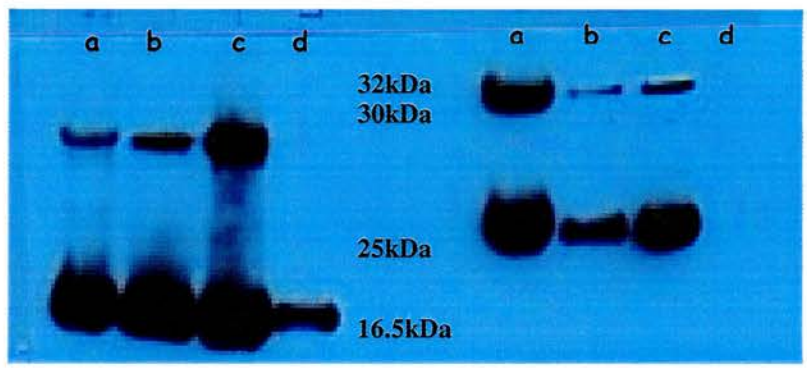


Figure 3.17b: Chemiluminescent western blot of pTrxFus ORF65 Ni-NTA purification. Ni-NTA purified pTrxFus-orf65 (10µl) was electrophoresed alongside Ni-NTA purified pTrxFus (pT), present as a control. The electrophoresed protein was analysed by western blotting using anti-HisTag antibody as a primary conjugate, and was visualised with chemiluminescence using the Pierce Supersignal ELISA femto HRP substrate. The western blot shows four stages of Ni-NTA resin; (a) represents the crude lysate (b) represents wash fractions (c) and (d) represents the first and third elution steps.

3.3.3. Review of OvHV-2 sequence

As the candidate genes initially selected could not be expressed due to showed solubility and purification problems, new candidates were sought that might prove more successful for protein expression and purification. The sequencing of the AlHV-1 and OvHV-2 genomes identified about 12 genes unique to the MCF viruses. Of these genes, four were unique to the OvHV-2 genome (Hart *et al.*, 2007) and may also be good candidates for developing OvHV-2 specific reagents. These genes were selected, since their protein products would be less likely to cross-react with other viruses. In addition, polypeptides likely to be exported or expressed on the cell surface were preferred since they might be more likely to be exposed to the immune system.

Gene	Length (bp)	Tm	% GC	Orientation	Product size (bp)	Sequence
Ov2.5	25	67.9	60	Forward	879	5'-**CCATGG**TCCTCCCCCTACGAGGTAA-3'
	26	66.4	53.8	Reverse		5'-**CCATGG**TGACCCCAAAGTAGCTTTCC-3'
Ov3.5	22	67.7	68.2	Forward	400	5'-**CCATGG**CCGCGGTAGACAGCTC-3'
	25	69.5	64.0	Reverse		5'-**CCATGG**CAGGTGGGGACTGTTGAGG-3'
Ov7	23	66.0	60.9	Forward	128	5'-**CCATGG**CCCTCTACAGGCAGCTA-3'
	24	66.0	58.3	Reverse		5'-**CCATGG**GGTAGCTGAACTCCACAC-3'
Ov8.5	20	63.5	65	Forward	1151	5'-**CCATGG**TGTCCCGGCTTCCT-3'
	23	66	60.9	Reverse		5'-**CCATGG**CAATAGACGGCGCCTGA-3'

Table 3.4: PCR Primer sequences. Primers were designed using Primer3, excluding transmembrane or signal sequences as defined by SignalP. All primers included terminal in-frame NcoI sites for cloning and gene fusion.

Using Primer3 (release 1.0: URL 1), primers were designed for OvHV-2 genes Ov2.5, Ov3.5, Ov7 and Ov8.5 (Tables 3.2 and 3.4), excluding predicted transmembrane or signal sequences in order to improve solubility of the expressed products. All sequences analysed showed 100% homology to the OvHV-2 sequence. Signal peptides were assigned using the signal peptide prediction server, SignalP (version 2.0: URL 2). PCR reactions were performed using the designed primers and genomic DNA purified from the infected cattle cell line BJ1035 (Hart *et al.*, 2007). Because Ov2.5 is a spliced gene, cDNA was used as the PCR template to provide a continuous open reading frame suitable for expression in bacterial systems (Figure

3.18c). Using the KOD Hot Start DNA polymerase (Novagen), PCR reactions were optimised for each primer pair by adjusting the magnesium ion concentration and the annealing temperature. A gradient PCR was performed, whereby temperatures ranging between 55.5 and 70°C were analysed for each gene product (Figure 3.18a & c). The optimal annealing temperature for Ov2.5, Ov3.5 and Ov7 was 60°C, while the optimal annealing temperature for Ov8.5 was 72°C. In addition four different magnesium concentrations were analysed, 0.5mM, 1.0mM, 1.5mM and 2.0mM (Figure 3.18b). The optimal magnesium concentration was 1.0mM for all amplifications.

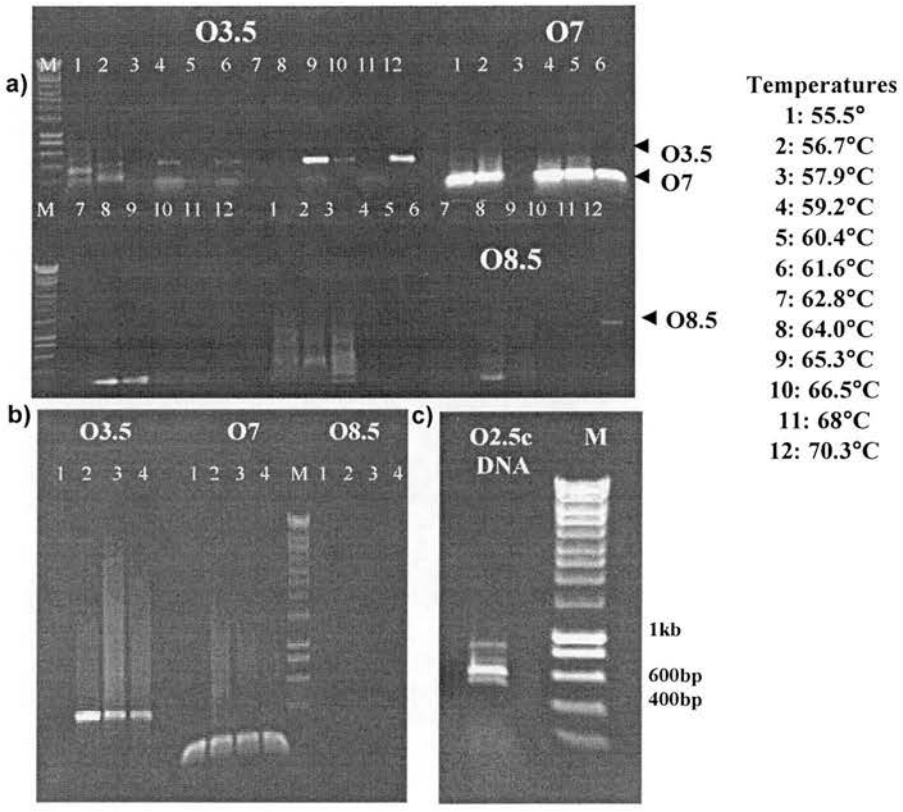


Figure 3.18: PCR Optimisation.

DNA (cDNA in case of Ov2.5) from the cell line BJ1035 was used as a template for PCR, using primers designed to amplify specific areas of Ov2.5 (463), Ov3.5 (400bp), Ov7 (128bp) and Ov8.5 (1151bp) gene sequences. Primers were optimised by performing (a) annealing temperature gradient PCR and (b) Mg^{++} titrations. cDNA from BJ1035 was used as a template for Ov2.5. Annealing temperatures investigated ranged from 55.5°C to 70.3°C, with increasing annealing temperatures from 1-12 for each gene. Mg^{++} concentrations analysed were 0.5mM (1), 1.0mM (2), 1.5mM (3) and 2.0mM (4). The PCR products were analysed by agarose gel electrophoresis alongside a 1kb DNA ladder (Hyperladder I, Bioline).

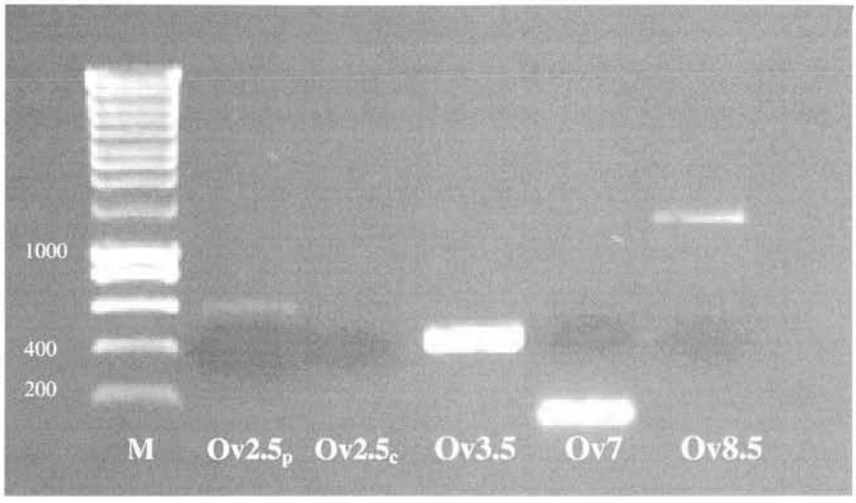


Figure 3.19: Gel Extraction of PCR products

OvHV-2 ORF Ov2.5 (Spliced: $Ov2.5_p$ and $Ov2.5_c$ represents two different spliced PCR products), Ov3.5, Ov7 and Ov8.5 purified PCR products. DNA samples were prepared using QIAGEN gel extraction and PCR purification techniques and concentrated by ethanol precipitation. The PCR products (10µl) were analysed by electrophoresis on a 0.8% agarose gel, alongside a 1kb DNA ladder (Hyperladder I, Bioline).

Following extraction the samples were concentrated by ethanol precipitation prior to ligation into the pGEM-T easy vector and transformation into JM109 competent cells. Plasmid minipreps of colonies with possible inserts were prepared and the presence of inserts was confirmed by restriction digestion with EcoRI. Of 32 colonies analysed in this way, one colony with $Ov2.5_p$ insert (partially spliced), 5 colonies with $Ov2.5_c$ insert (completely spliced), 11 colonies with Ov3.5 insert, 3 colonies with Ov7 insert and 11 colonies with Ov8.5 insert were obtained (Figure 3.20).

Ten colonies were sequenced of which: 1 colony represented $Ov2.5_c$, 1 colony represented Ov7; and 4 colonies each represented $Ov2.5_p$ and Ov3.5 (highlighted in figure 3.20). Sequencing results confirmed the presence of insert without any PCR errors in all colonies analysed.

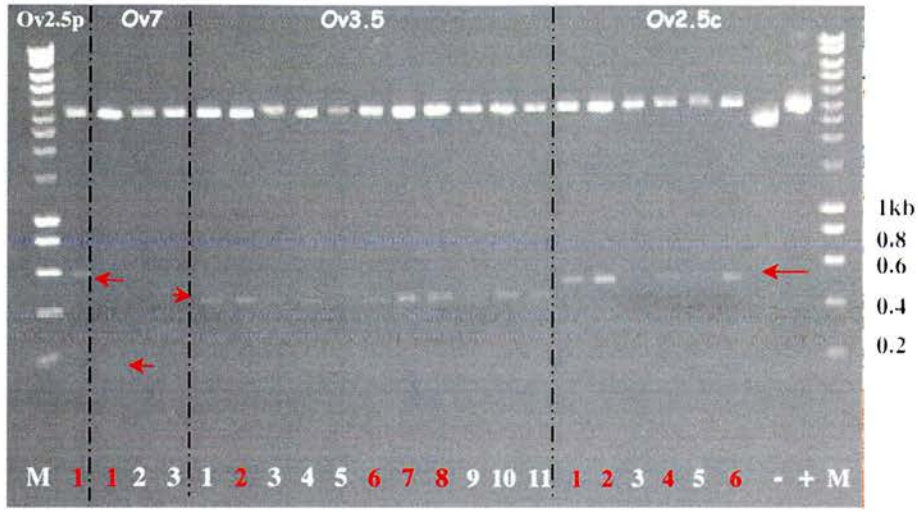


Figure 3.20: DNA miniprep analysis

DNA prepared from bacterial clones using a QIAprep spin miniprep kit. The DNA was digested with *Eco*RI and the products electrophoresed on a 0.8% agarose gel alongside a DNA marker (M). For Ov2.5 clones Ov2.5p represents partially sliced insert and Ov2.5c represents completely spliced insert. Highlighted numbers indicate colonies selected for sequencing. Positive (+) and negative (-) TA-cloning controls were also included.

3.3.4. pTrxFus OvHV-2 Ov2.5, Ov3.5, Ov7 & Ov8.5

NcoI-digested insert DNA from OvHV-2 Ov2.5, Ov3.5, Ov7 and Ov8.5 clones was ligated into the pTrxFus expression vector. Plasmid DNA carrying verified inserts were transformed into *E-coli* G1724, the expression host for this vector. Cultures of each clone were grown to an OD_{550} of 0.5 at 30°C then induced by the addition of 100µg/ml tryptophan. Cells were harvested four hours after induction. Samples of induced cultures were lysed in SDS loading buffer and 10µl of each lysate was electrophoresed on a 10% NuPAGE bis-tris polyacrylamide gel. The gel was stained with Coomassie blue stain (Figure 3.21). No obvious bands for any of the genes; pTrxFus-Ov2.5, pTrxFus-Ov3.5, pTrxFus-Ov7 or pTrxFus-Ov8.5 were distinguished, indicating no protein over-expression.

To identify whether pTrxFus-Ov2.5, pTrxFus-Ov3.5, pTrxFus-Ov7 and pTrxFus-Ov8.5 were expressed in the crude lysates, the electrophoresed protein lysate was analysed by western blotting using anti-HisTag antibody as a primary antibody followed by anti-mouse HRP conjugate (Figure 3.21b). The pTrxFus vector

expressed a single His-tagged band at approximately 16.5kDa, the size expected for thioredoxin, pTrxFus-Ov2.5 expressed a band at approximately 36kDa (expected size of 34kDa) and pTrxFus-Ov7 expressed a single band at 22kDa (expected size of 22kDa). In contrast pTrxFus-Ov3.5 and pTrxFus-Ov8.5 showed no expression using an anti-HisTag monoclonal antibody.

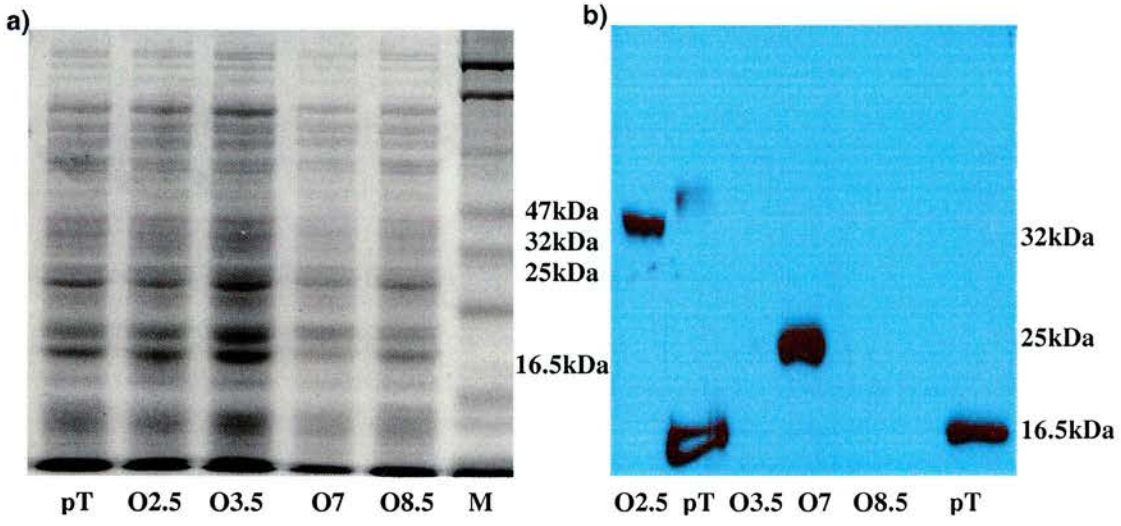


Figure 3.21: Expression of OvHV-2 genes in the thiofusion expression system. a) Coomassie blue stained 10% NuPAGE® Novex bis-tris gel, showing thiofusion expression system cultures pTrxFus-Ov2.5, pTrxFus-Ov3.5, pTrxFus-Ov7 and pTrxFus-Ov8.5 and the pTrxFus vector, four hours after induction with Tryptophan. b) 20µl of whole cell lysate pTrxFus-Ov2.5, pTrxFus-Ov3.5, pTrxFus-Ov7 and pTrxFus-Ov8.5 analysed by western blotting using anti-HisTag antibody as a primary antibody followed by anti-mouse HRP conjugate and chemiluminescense. pTrxFus empty vector (pT) present as a control.

To define whether pTrxFus-Ov2.5 and pTrxFus-Ov7 were soluble, cultures were mechanically lysed and a western blot was performed on soluble and pellet fractions using an anti-HisTag monoclonal antibody (Figure 3.22). The pTrxFus vector expressed 2 His-tagged bands, at approximately 16.5kDa, the size expected for thioredoxin, and at approximately 36kDa in both the soluble and insoluble fractions. pTrxFus-Ov2.5 showed no protein expression in the soluble phase although expressed His-tagged bands at approximately 36kDa and 64kDa were seen in the pellet. pTrxFus-Ov7 also showed no expression in the soluble phase but expressed bands at approximately 25kDa and 50kDa in the insoluble cell pellet.

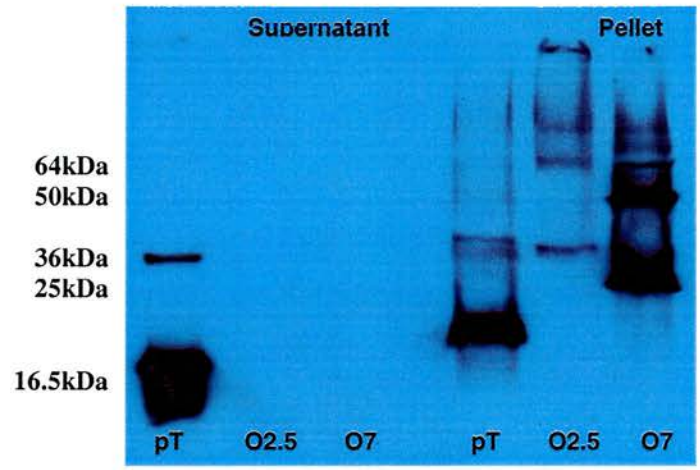


Figure 3.22: Chemiluminescent western blot of soluble and insoluble pTrxFus-Ov2.5 and pTrxFus-Ov7. Soluble and insoluble (10µl) pTrxFus-Ov2.5 (Ov2.5) and pTrxFus-Ov7 (Ov7) extracts were electrophoresed alongside a pTrxFus extract control (pT). The electrophoresed protein was analysed by Western blotting using anti-HisTag primary antibody.

3.3.5. Analysis of the Ov8 Exon 1 0.5kb construct

The Ov8 gene fragment expressed in section 3.3.1.2.2 was based on an early interpretation of the OvHV-2 genome, which indicated a 2.1kb Ov8 exon1. The final annotation of OvHV-2 (Hart *et al.*, 2007) has altered intron/exon boundaries that suggest Ov8 exon 1 is only 1.2kb (Figure 3.23a). The remaining 0.8kb of the previously cloned ORF may include sequences that are intronic. This may be the cause of problems found expressing the Ov8 construct and its insolubility in the pET expression system.

New primers were designed to amplify the newly-defined OvHV-2 Ov8 (Table 3.5), by the method described earlier (section 3.2.1.1), to exclude transmembrane and signal sequences, which have low hydrophilicity. PCR reactions were performed using genomic DNA purified from the infected cattle cell line BJ1035. The Ov8 sequence showed 100% homology with the OvHV-2 sequence. Although the Ov8 gene is 1.2kb, a 531bp fragment, representing Ov8 exon 1 was amplified, excluding regions of low hydrophilicity (Figure 3.23b). PCR reactions were optimised for each primer pair by altering the magnesium ion concentration and the annealing temperature. The optimal annealing temperature for Ov8 exon 1 was 60°C.

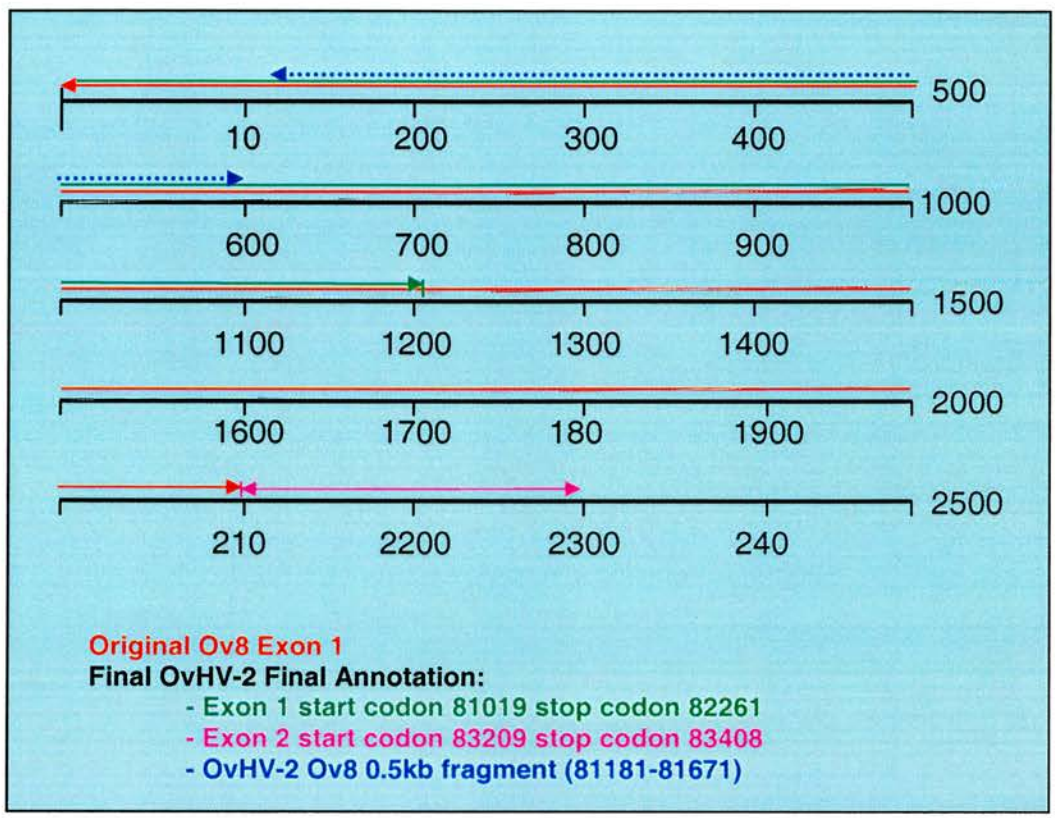
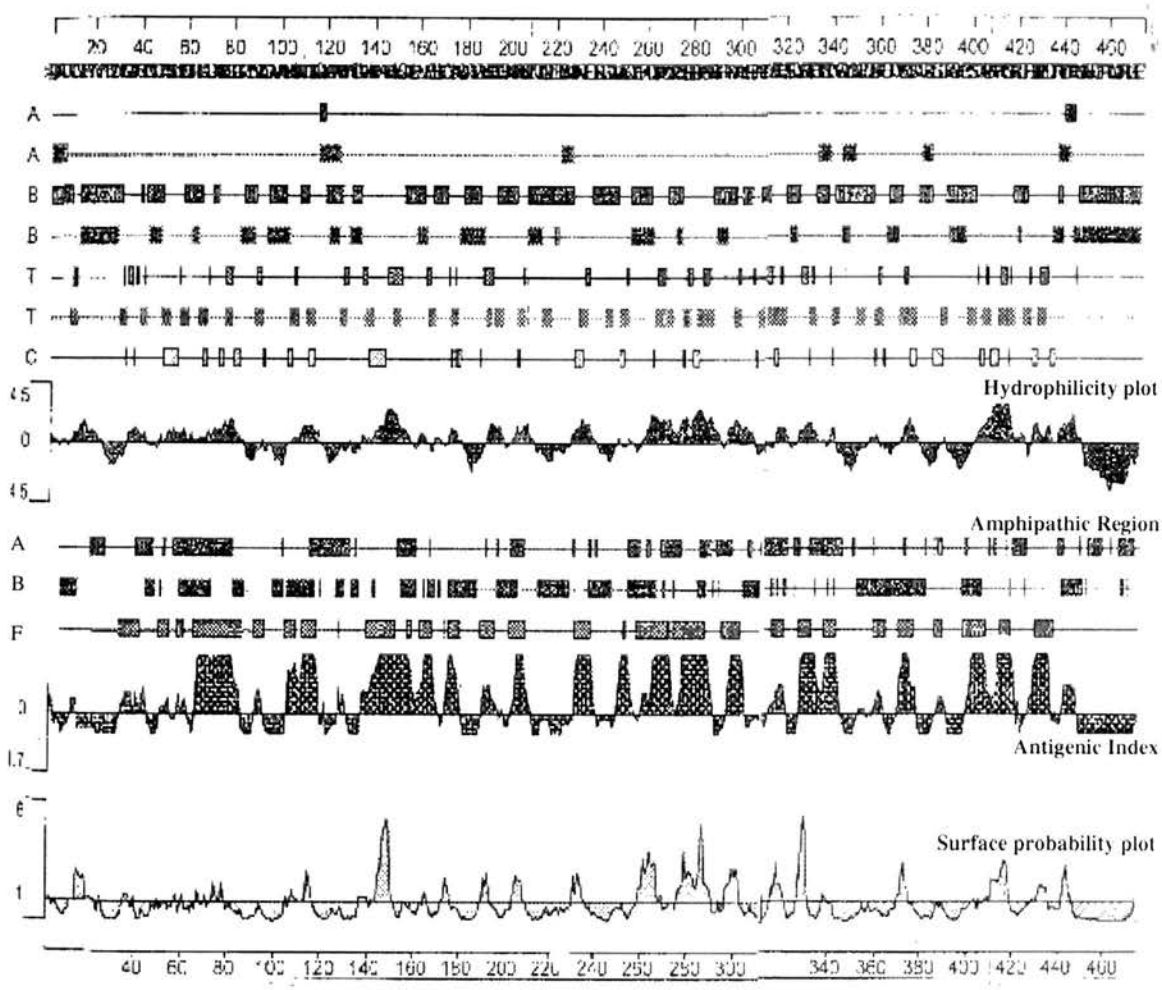


Figure 3.23a: Annotation of Ov8 sequence. The original Ov8 primers were designed using an earlier annotation of the OvHV-2 sequence. The original primers designed amplify a 2.1kb fragment highlighted in red. The published OvHV-2 shows a change in the splice site resulting in a 1.2kb fragment (highlighted in green). The primers designed for Ov8 Exon 1 (0.5kb) is highlighted in blue.



A – Alpha regions, B- Beta regions, T – Turn regions, C – Coils, F – Flexible regions

Figure 3.23b: Hydrophobic regions of the OvHV-2 Ov8 sequence. Plot was performed by DNAstar PROTEAN using the published OvHV-2 sequence taken from Genbank. The plot shows regions of hydrophilicity (Kyte-Doolittle), amphipathic regions and areas of protein folding. The hydrophobicity plot was performed to analyse the best area of Ov8 Exon 1 to amplify, whereby solubility issues may be resolved.

Gene	Length (bp)	Primer Coordinates	Tm	% GC	Sequence
Ov8For	33	81640-81673	75	72.7	5'-**GGCGCGCCCATGG**GGCCTGCGTACCCCAACTGT-3'
Ov8Rev	36	82272-82308	75	69.4	5'-**GGCCGGCCCCATGG**GCACGTTGGTCAGTCGGTTAGG-3'

Table 3.5: Ov8 Exon 1 0.5kb PCR Primer sequences.
Primers were designed using Primer3, excluding transmembrane or signal sequences as defined by SignalP. All primers included terminal in-frame NcoI sites for cloning and gene fusion. Ov8 Exon 1 (0.5kb) forward and reverse primer used to amplify a 531bp product. Non-virus sequences are highlighted.

3.3.5.1. Cloning PCR products with pGEM®-T vector

OvHV-2 Ov8 Exon 1 0.5kb was purified directly from the PCR reactions as little background amplification was found. 10µl of the purified PCR product was electrophoresed to confirm recovery (Figure 3.24).

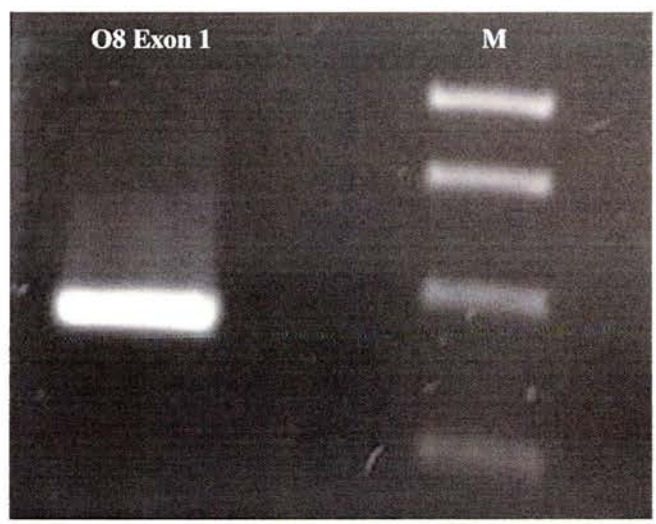


Figure 3.24: pTrxFus-Ov8 Exon 1 PCR product. Product was electrophoresed on a 0.8% agarose gel alongside a DNA marker (Hyperladder I, Bioline).

Following extraction the DNA was concentrated by ethanol precipitation prior to ligation and transformation into JM109 competent cells as described in section 2.4.5. Plasmid minipreps of colonies with possible inserts were prepared and the presence of insert was confirmed by restriction digestion with NcoI. Clones with

insert of the correct size were sequenced to confirm the identity of the insert and exclude any clones with PCR errors.

3.3.5.2. OvHV-2 Ov8 Exon 1 0.5kb in the pTrxFus Vector

Insert DNA from a sequence-confirmed clone of OvHV-2 Ov8 Exon 1 0.5kb was transferred into the pTrxFus expression vector (as described for other gene fragments sections 3.2.2. and 3.2.2.3), prior to transformation into *E. coli* GI 724. Cultures of Ov8 Exon 1 0.5kb clone and empty pTrxFus vector were grown and induced by the addition of tryptophan. Cells were harvested up to four hours after induction. Samples of induced cultures were lysed in SDS loading buffer and 10µl of each lysate was electrophoresed on a 12% NuPAGE bis-tris polyacrylamide gel. The gel was stained with SimplyBlue SafeStain (Figure 3.25a), and was analysed by western blotting using HisProbe-HRP antibody (Figure 3.25b). No obvious overexpression of pTrxFus-Ov8 Exon 1 0.5kb was seen by staining, but western blotting visualised pTrxFus-Ov8 Exon 1 0.5kb as two bands close to 30kDa, the size expected for the thioredoxin Ov8 fusion. As in the pET system (Section 3.3.1.2.), the two bands may be a result of the protein being cleaved into two stable products. As both constructs carry the N-terminal region of Ov8, the same cleavage site could be responsible.

To test whether pTrxFus-Ov8 Exon 1 0.5kb protein was soluble, cultures were mechanically lysed and soluble and insoluble fractions were analysed by staining and western blotting (Figure 3.25c). Similarly to the crude lysate, pTrxFus-Ov8 Exon 1 0.5kb soluble lysate showed two bands at approximately 30kDa. No Ov8 fusion protein was detected in the cell pellet.

3.3.5.3. Purification under Denaturing conditions (8M Urea)

To isolate the target pTrxFus-Ov8 Exon 1 0.5kb His-tagged protein, affinity purification was performed. Purification of the pTrxFus-Ov8 his-tagged crude lysate was attempted using a HisTrap nickel affinity purification column (GE Healthcare). Proteins containing the His-tag sequence should bind to cross-linked agarose beads with immobilized nickel under native or denaturing conditions.

As native conditions proved unsuitable for the purification of the protein fusions tested, denaturing conditions were employed using 8M urea in all buffers. To confirm the presence of the His-tagged proteins, eluted fractions were pooled, concentrated 30-fold and analysed by western blotting using HisProbe-HRP. The

concentrated eluant showed no Ov8 fusion, suggesting that the His-tagged proteins did not bind to the column (Figure 3.26). However, the unbound material was also analysed alongside the eluant and again no Ov8 fusion protein was detected.

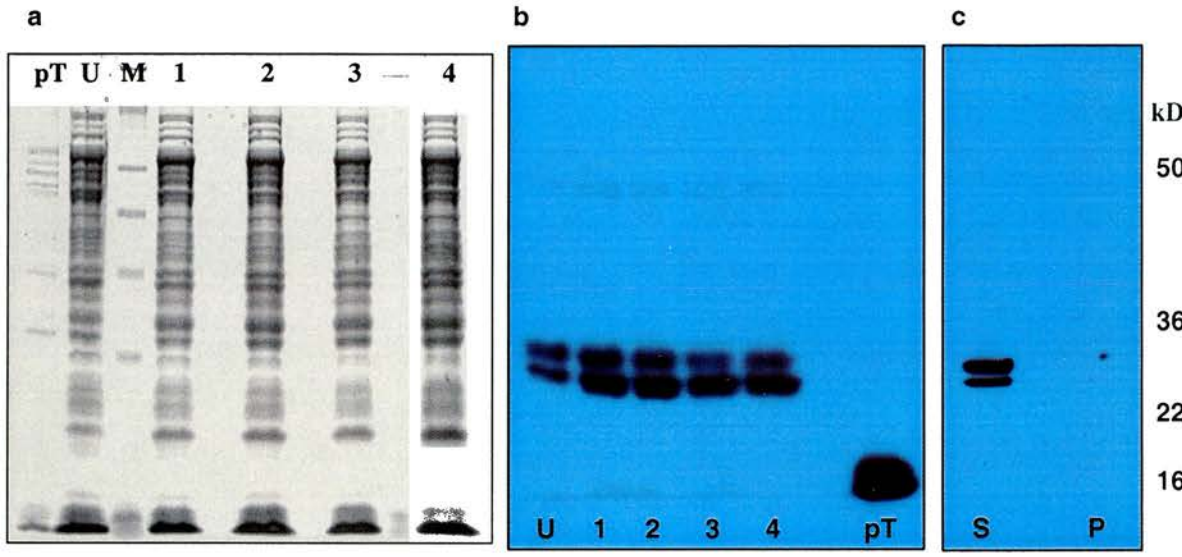


Figure 3.25: Electrophoresis of pTrxFus-Ov8 (Exon 1) crude lysate. a) Coomassie blue stained 12% NuPAGE® Novex bis-tris gel, showing Thiofusion Expression system cultures pTrxFus-Ov8 (Exon 1) at hourly intervals for four hours after induction with Tryptophan (1-4). M represents the protein marker. U represents uninduced samples. (b) 20µl crude lysate of pTrxFus-O8 Exon 1 were electrophoresed alongside an empty vector control (pT). The electrophoresed protein lysate was analysed by Western blotting using HisProbe-HRP. (c) 20µl of soluble (S) and insoluble pellet (P) pTrxFus-Ov8 Exon 1 extracts (Eton-press) were electrophoresed and analysed by Western blotting using HisProbe-HRP.

Furthermore, no protein was found in either the unbound material or eluent by staining using SimplyBlue SafeStain. This may reflect the low level of protein in the extract as a result of a dilution effect caused by the purification procedure or by the loss of protein during purification. It was not possible to check for irreversible binding to the column by analysing the resin.

3.3.5.4. Gel filtration/purification of pTrxFus Ov8 Exon 1 0.5kb

Since the initial HisTrap purification of Ov8 Exon 1 0.5kb fusion protein proved ineffective, a further purification technique was investigated. Gel filtration was used to fractionate crude lysates containing His-tagged fusion proteins according to size. Separation was performed on a $Superdex_{200}$ column, using the Akta FPLC

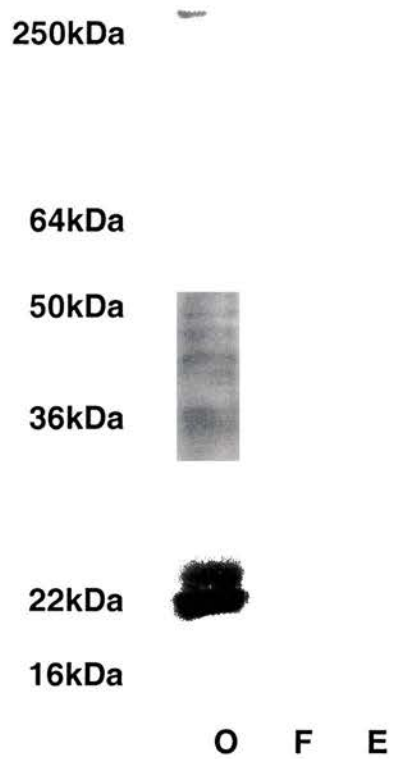


Figure 3.26: Chemiluminescent western blot of pTrxFus-Ov8 (Exon 1, 0.5kb) gene product. Gel electrophoresis of solubilised pTrxFus-Ov8 Exon 1 (subsequent to purification via HisTrap affnity purification) performed on a 12% tris-glycine polyacrylamide gel, and analysed by western blot using HisProbe-HRP. O represents the orignal Ov8 supernatant, F represents the fall-through material from the HisTrap column and E represents the eluant.

system. Five hundred microlitres of pTrxFus-Ov8 Exon 1 0.5kb soluble phase collected following mechanical lysis was injected onto the column and 1ml fractions were collected and analysed. Thirty fractions were collected and fractions which corresponded to protein peaks on the FPLC system UV-trace were analysed by Coomassie staining and western blotting using HisProbe-HRP antibody (Figure 3.27) to detect the presence of pTrxFus-ORF Ov8 Exon 1 0.5kb protein in the eluted fractions. The stained gels showed protein in all of the peak fractions, with apparent molecular weights ranging from 14kDa to 97kDa. The initial peak, corresponding to fractions 7 and 8, showed a single protein band at 64kDa. The second peak, corresponding to fractions 12, 13 and 14, showed a high level of eluted proteins, while fractions corresponding to the third fractionation peak (fractions 15/17)

showed the majority of proteins ranged from 14 to 64kDa, and had a distinct band at approximately 30kDa, the size expected for the Ov8 exon 1 protein fusion (Figure 3.27a).

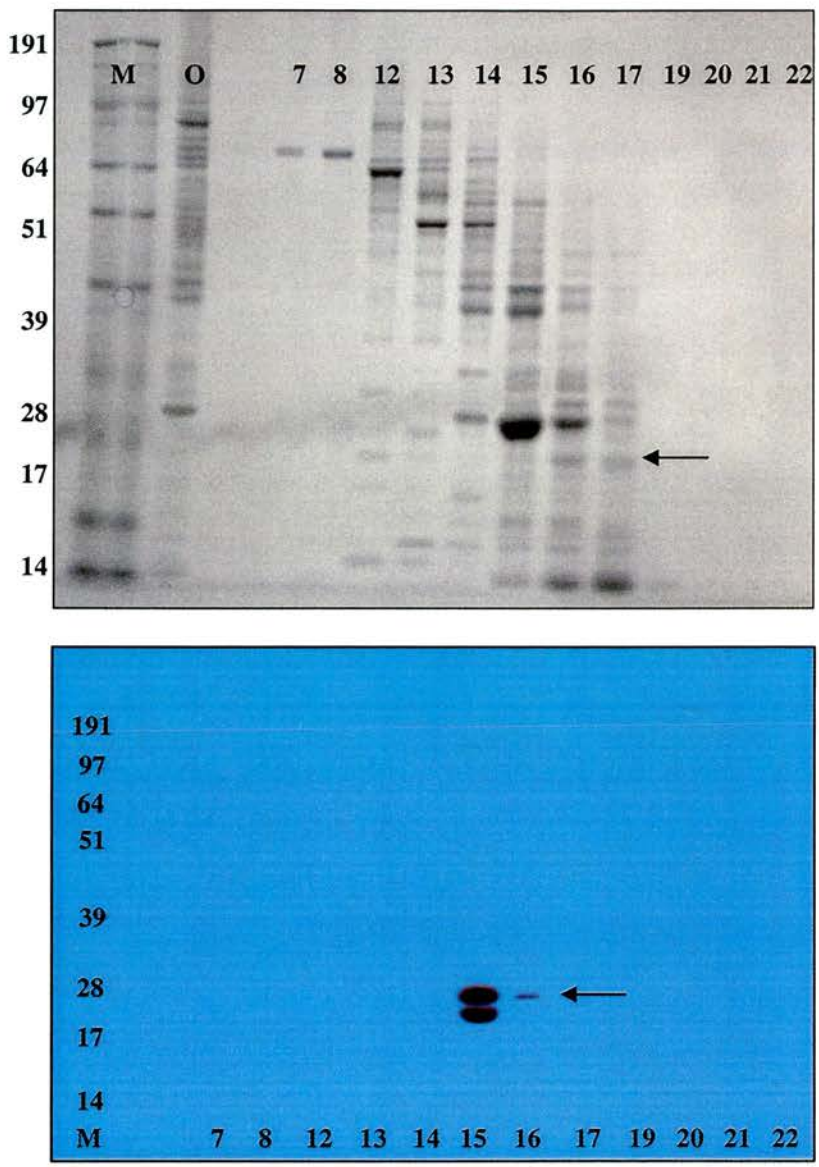


Figure 3.27: Gel filtration of pTrxFus-Ov8 Exon 1. a) SimplyBlue Safestain stained 12% NuPAGE® Novex bis-tris gel, showing 15µl fractions of the Thiofusion Expression system culture pTrxFus-Ov8 (Exon 1) after gel filtration using the $Superdex_{200}$ column. M represents the protein marker. O represents original Ov8 crude lysate. b) 15µl Fractions (concentrated two-fold) of pTrxFus-O8 Exon 1 protein lysate was analysed by Western blotting using HisProbe-HRP. The arrow indicates a protein of 30kDa, which may represent the Ov8 protein.

A western blot, using HisProbe-HRP antibody, of the same fractions detected expression of two His-Tagged bands, at approximately 30kDa in size, present in the third peak (fractions 15 & 16), corresponding to bands shown by staining (Figure 3.27b). As the Ov8 Exon 1 0.5kb fusion protein appeared to correspond to a stained band, the fraction containing the detected band was analysed by western blotting using crude MCF positive and negative sera and IgG purified from these same sera (Figure 3.28).

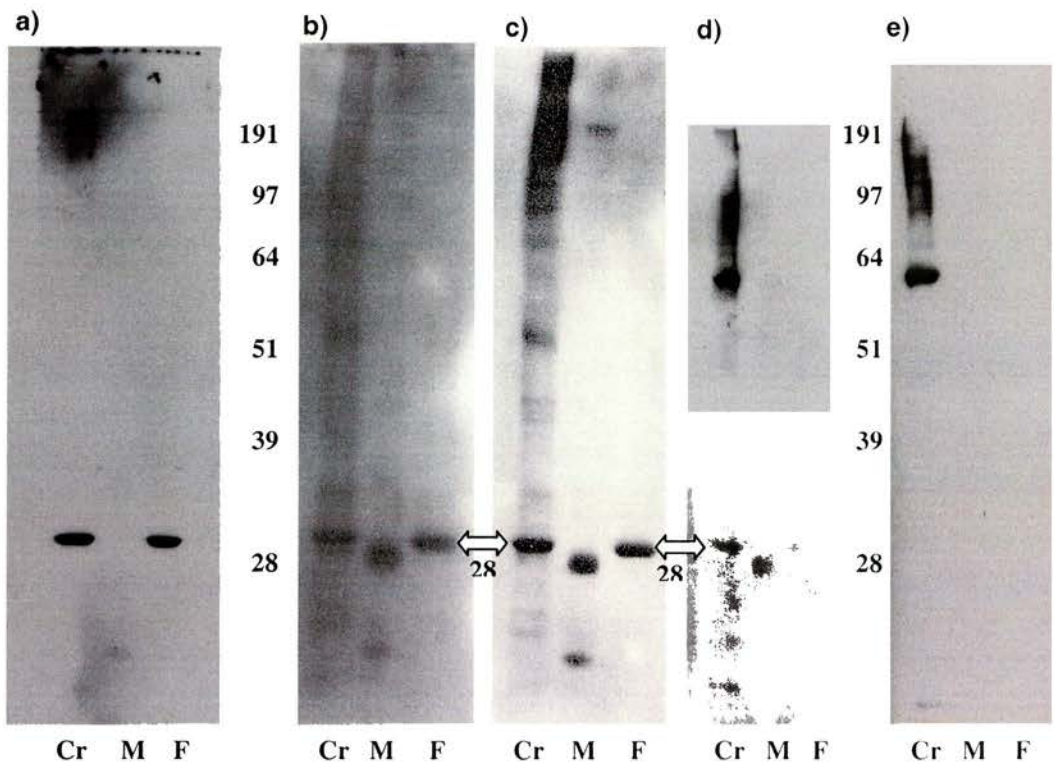


Figure 3.28: Chemiluminescent analysis of pTrxFus-Ov8 (Exon 1) with MCF sera. Fractions (concentrated two-fold) of pTrxFus-Ov8 Exon 1 protein lysate (fraction 15, Figure 1.2.21) was analysed by western blotting using a) HisProbe-HRP b) MCF-positive crude serum c) MCF-positive IgG purified serum and d) MCF-negative crude and e) MCF-negative IgG purified serum, followed by rabbit anti-bovine HRP conjugate. Cr represents crude pTrxFus-Ov8 (exon 1) lysate, F represents the concentrated pTrxFus-Ov8 fraction (fraction 15) while M represents the protein marker (SeeBlue Plus2 Protein marker, Sigma). The arrows indicate a protein of 30kDa, conserved in MCF-positive and negative serum.

Both positive and negative crude serum detected a low molecular band and a range of high molecular weight bands (50-200kDa) in the crude extract. The MCF-positive and -negative crude serum detected a 30kDa band in both the crude and gel-filtered protein fractions. This result shows the 30kDa protein was detected

by both MCF-positive and -negative crude serum, and suggests that some component of the Ov8-thioredoxin fusion may be recognised by both sera. This may be a direct result of thioredoxin itself, as thioredoxin is an *E. coli* protein. As thioredoxin is a bacterial protein, it is possible that cattle sera have antibodies to bacterial thioredoxin. Thioredoxin may therefore be a poor fusion partner for this purpose.

Purification of IgG fractions from the crude MCF positive and negative sera showed differences in protein degradation. The presence of the 30kDa band which reacted with the IgG purified MCF-positive and –negative sera, in both crude and gel-filtered protein fractions, suggests that the band identified would not be OvHV-2 specific due to being present in the control. Further experiments therefore need to be performed for the continued recognition of the Ov8 protein. Additionally, a larger pool of MCF-positive and negative crude, and IgG purified sera should be tested to confirm this finding.

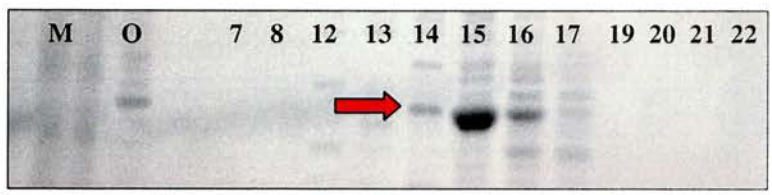


Figure 3.29: Mass spectrometry band analysis of the fractionated pTrxFus-Ov8 Exon 1 (fraction 15). Match to: SODM_ECO57 Superoxide dismutase Taxonomy: *Escherichia coli,* Sequence coverage of 55%. Matched peptides are shown in red (Bold). The band sequenced is idicated by the arrow.

```
  1 SYTLPSLPYA YDALEPHFDK QTMEIHHTKH HQTYVNNANA ALESLPEFAN
 51 LPVEELITKL DQLPADKKTV LRNNAGGHAN HSLFWKGLKK GTTLQGDLKA
101 AIERDFGSVD NFKAEFEKAA ASRFGSGWAW LVLKGDKLAV VSTANQDSPL
151 MGEAISGASG FPILGLDVWE HAYYLKFQNR RPDYIKEFWN VVNWDEAAAR
201 FAAKK
```

The most abundant band from fraction 15 (Figure 3.27) was analysed by mass spectrometry (Figure 3.29). It was not certain that the purified product was the expressed Ov8 Exon 1 0.5kb protein, although the expressed protein was of the same size as expected for the Ov8 Exon 1 0.5kb protein. The protein match identified *E. coli* (0157:H7) superoxide dismutase, showing 55% sequence similarity. Although the major protein was not Ov8, it is possible that the Ov8 fragment is expressed at a lower level and is therefore masked behind the superoxide dismutase. This confirms the observation that induction of Ov8 expression did not give rise to an over-expressed band visible by Coomassie blue staining.

3.4. Discussion

Current diagnosis of MCF is reliant on an indirect immunofluorescence test (IIF) and a PCR assay. Although there are ELISAs commercially available (Wan *et al.*, 1988, Li *et al.*, 2001), they maybe of limited value in the detection of OvHV-2 specific antibodies, as MCF viruses are known to share antigens with other herpesviruses. This may result in a general insensitivity or non-specificity that may limit the test (Li *et al.*, 1994).

The recent determination of the DNA sequence of the OvHV-2 genome has allowed about seventy genes to be identified, of which twelve are unique to the MCF viruses and three are unique to OvHV-2 (Hart *et al.*, 2007). Consequently, these genes may prove good candidates for the development of specific assays for detection of OvHV-2 infection. The aim of this work was to clone and express OvHV-2 genes as recombinant proteins, which may be used to establish an ELISA for the detection of anti-MCF antibodies in field serum. In order to determine whether the recombinant proteins can be detected by antibodies in sera from MCF-affected animals, purification is required to eliminate non-specific cross-reactivity with components from bacterial expression systems such as *E. coli.*

Initial studies showed expression of the OvHV-2 genes Ov8 Exon 1 2.1kb and ORF65 in bacterial expression systems; pBAD, pET-22b and pTrxFus. Expression of OvHV-2 proteins from pTrxFus-Ov8 Exon 1 2.1kb, pTrxFus-ORF65 and pET22b-Ov8 Exon 1 2.1kb were demonstrated by western blotting using anti-HisTag and anti-Thio antibody.

In the pBAD expression system, pBAD-Ov8 Exon 1 2.1kb and pBAD-ORF65 were detected in the crude lysate, but no expression was detected for pBAD-Ov9. These results, suggest a possible problem with the pBAD-Ov9 protein expression under the conditions used and expression system employed. It could also indicate a genetic problem, whereby the insert suffers rearrangement when cloned into the expression vector, although no evidence for this was detected. However, Ov9 had previously been impossible to clone into the pTrxFus vector.

pBAD-Ov8 Exon 1 2.1kb expressed two bands at 80kDa and 100kDa rather than the expected single, 80kDa band. This may indicate specific protein degradation into two stable products.

In the pET-22b expression system, only pET22b-Ov8 Exon 1 2.1kb could be detected, while no expression was detected for pET22b-Ov9 or pET22b-ORF65.

These results suggest a possible problem with either the pET22b-Ov9 and pET22b-ORF65 protein expression under the conditions used and expression system employed. While Ov8 was expressed in this system, the protein was insoluble and hence was not investigated further.

Although it appeared possible to clone Ov9 into the expression plasmids, it was not possible to express in any system. Russell and Wiyono (unpublished data) demonstrated plasmid rearrangement in pTrxFus and, although it was not investigated in the constructs made here, it is possible that arrangements also occurred that prevented expression in pET22b and pBAD. Alternatively, since the cloned fragments of Ov9 included the hydrophobic C-terminal transmembrane region, this may have resulted in problems with protein folding and consequent stability.

Herpesvirus virions are surrounded by an icosahedral protein capsid comprised of capsid proteins indicating ORF65. OvHV-2 ORF65 shares homology with human herpesvirus-8 ORF65, which has been utilised in a serological assay for the diagnosis of Kaposi's sarcoma (Perez *et al.*, 2006). HHV-8 ORF65 has been cloned and expressed as GST fusion proteins in bacterial and baculovirus expression systems (pGEX-4T-1) (Zhu *et al.*, 1999). Expression in the expression vector pQE-42 has also been demonstrated (Simpson *et al.*, 1996). Due to the structure and function of OvHV-2 ORF65, aggregation may occur causing the protein to remain insoluble, but these other systems may offer an alternative method for the successful expression of OvHV-2 ORF65.

The expression vector pET-22b encodes a 21aa bacterial leader sequence, which targets expression into the bacterial periplasm. Rather than the expected single, 80kDa pET22b-Ov8 Exon 1 2.1kb band two bands at 80kDa and 100kDa were observed. A possible explanation for this 20kDa difference might have been that cleavage of the leader peptide sequence present in the pET-22b expression system occurred during export into the periplasm. The difference in migration however, is much bigger than that expected from signal peptide cleavage, and for this explanation to be true the presence of the leader peptide would have to significantly influence the migration of the protein. An alternative explanation may be that the protein underwent specific proteolytic cleavage at a different site at the N-terminus of the expressed Ov8 Exon 1 2.1kb protein.

The pET22b-Ov8 Exon 1 2.1kb protein was insoluble after mechanical lysis. Furthermore, it did not appear to be solubilised from the pellet in the presence of concentrated urea. This result may be due to problems in this western blot procedure, as suggested by the high background. Further possibilities include very poor solubility or aggregation of the pET22b-Ov8 Exon 1 2.1kb product. Therefore further studies should be performed on the pET22b-Ov8 Exon 1 2.1kb product to clearly define its solubility.

High levels of expression of recombinant proteins in a bacterial system can lead to the formation of insoluble aggregates, or inclusion bodies. Inclusion bodies are dense aggregates of misfolded polypeptide. They are formed intracellularly as a result of either aggregating characteristics of the protein or insufficient cellular processes which ensure expressed polypeptides are soluble and folded correctly. Inclusion bodies are usually located in the bacterial cytoplasm, though secreted proteins may form inclusion bodies in the periplasmic space. Some proteins, which are incorporated into inclusion bodies, may possess elements of native structure, and therefore, must be released from the inclusion body and refolded to give its native structure (Middelberg, 2002). To solubilise these inclusion bodies, strong denaturing agents such as 6M Guanidine-HCl (GuHCl) and 8M Urea can be used. Therefore, to test whether pET22b-Ov8 could be solubilised strong denaturing agent, urea (8M), was used to solubilise the whole cell crude lysate.

Additional trials could be performed in order to improve solubility of this protein. Possible trials include the growth of bacterial cells at a lower temperature to improve protein solubility and an IPTG titration to determine the optimal concentration required to induce the pET-22b expression system.

Work to clarify the difficulty with the Ov8 Exon 1 2.1kb construct in the expression vector pTrxFus led to the suggestion that the fragment of the Ov8 gene used in initial expression studies contained some intron sequence. The latest analysis of the OvHV-2 genome sequence (Hart *et al.*, 2007) indicated that the cloned OvHV-2 Ov8 Exon 1 2.1kb fragment actively contained about 800bp of intron. The expression of an extended piece of coding intron may account for the insolubility of the protein product. Therefore a smaller, 531bp fragment encoding the N-terminal half of the newly annotated Ov8 Exon 1 0.5kb, was designed to test expression and solubility. Western blotting revealed the pTrxFus-Ov8 Exon 1 0.5kb fusion protein could be detected in the soluble fraction. However, similar to the expression studies on the original Exon 1 2.1kb Ov8 fragment in the pET system, the new Ov8 Exon 1

0.5kb construct gave rise to two bands at about 30kDa, indicating a conserved cleavage site in both Ov8 constructs. To isolate the target pTrxFus-Ov8 Exon 1 0.5kb His-tagged protein metal-affinity purification was performed under denaturing conditions. No protein was detected suggesting that the His-tagged proteins did not bind to the column. Therefore, as metal-affinity purification methods had also proved unsuccessful for the purification of pTrxFus-ORF65, alternative methods for purification were sought. The lysate was fractionated using a Superdex gel filtration column. This showed an enrichment of a 30kDa protein band the expected size of the Ov8 Exon 1 0.5kb product which was detectable by Coomassie staining and western blotting with the anti-his antibody. When tested using MCF-positive and -negative crude serum, a low molecular band at 30kDa was detected in both the crude extract and gel-filtered protein fraction. This result suggests that the 30kDa band may possibly represent forms of the thioredoxin fusion protein and therefore may be recognised by both sera. Further experiments may be required to separate recognition of Ov8 from that of the thioredoxin fusion partner.

The attempt to express the Ov8 gene fragment in order to overcome solubility issues proved successful. By analysing the hydrophobicity plot, primers could be designed to exclude not only regions of transmembrane and signal sequence, but regions of protein folding and hydrophobicity. By performing these plots and sequence analysis prior to the development of primers, solubility issues in expression systems may be eliminated.

When tested using IgG purified MCF-positive and negative serum, there was enrichment of the 30kDa band in the positive sera, while the band was absent in the negative sera. This result, contrasting to the tested crude serum, suggests the Ov8 may be an antigenic protein recognised by anti-MCF antiserum. This experiment would have to be repeated with a larger selection of crude and IgG purified serum to substantiate this finding.

Thioredoxin is a heat-stable redox protein, 12kDa, universally expressed in yeast, plants, mammals and bacteria. It was originally isolated from yeast extracts and subsequently from *E. coli*. In *E. coli* it acts as a hydrogen donor for ribonucleotide reductase, an enzyme which controls the cellular concentration of deoxyribonucleotides required for DNA synthesis. Mammalian thioredoxin has a variety of biological functions in cell growth, transcription and immune function (Matsui *et al.*, 1996) as well as acting as hydrogen donor for ribonucleotide reductase. It is therefore possible that most cows recognise bacterial thioredoxin, and

therefore, cross-react with the fusion proteins expressed. A more likely explanation however is that the crude bovine serum sample contains a number of proteins besides host proteins which may result in cross-reactivity.

This explanation could be confirmed by cleavage of the thioredoxin fusion to release the Ov8 Exon 1 0.5kb fragment from the MCF gene of interest. Western blots using sera should show whether the Ov8 fragment is recognised by both MCF-positive and -negative serum and whether thioredoxin is also recognised by both serum samples.

Another explanation may be that the bands that are being detected are not specific to the MCF gene but may represent a co-purified protein from the *E. coli* used for culturing. This is unlikely as the His-tag bands are right in the same place. In fact some *E. coli* proteins are detected but these are near the top of the gel. The band was subjected to mass spectrometry. A protein match was identified as *E. coli* superoxide dismutase, showing 55% sequence similarity. Although this suggests the abundant band at 30kDa was not the pTrxFus-Ov8 Exon 1 0.5kb fusion, it is possible that the Ov8 fragment is masked by the superoxide dismutase and was not detected due to its lower protein concentration. However, due to time constraints, further work on this was not performed to enable identification of the Ov8 Exon 1 0.5kb fragment although from this expression study, Ov8 appears the best possibility for a recombinant protein based diagnostic assay.

Work with the expression vector pTrxFus, revealed that the pTrxFus-ORF65 fusion protein could be detected in the soluble fraction, while pTrxFus-Ov8 Exon 1 2.1kb showed a band of the same size as the thioredoxin protein (16kDa). As the pTrxFus-ORF65 crude extract contained a soluble, His-tagged protein of 32kDa, the expected size for the ORF65 fusion, purification using a nickel resin was attempted. The lack of success in purification may have reflected the low expression level of the pTrxFus-ORF65 protein or poor affinity for the resin, possibly also affected by poor accessibility of the tag.

This initial finding for Ov8 suggested either a problem with the Ov8 construct or proteolysis of the fusion protein. Restriction analysis, however, showed that the correct construct structures were present in all isolates tested. Explanations for the discrepancy may relate to low expression levels, conditions used for protein expression or the degradation of the pTrxFus-Ov8 Exon 1 2.1kb fragment into smaller, stable products of similar sizes. Degradation, being the most likely

explanation, was substantiated by the detection of a weak 25kDa band and a 16kDa band present in the elution samples from Ni-NTA purification.

The expression of recombinant proteins in *Escherichia coli* is a routinely used method for the recovery of large amounts of mammalian proteins, however culture conditions and induction of expression may cause inhibitory effects to the yield of soluble, active proteins produced. Such effects may be frequently caused by protein misfolding, degradation and aggregation (Hanning and Makrides 1998). Numerous approaches have been used to improve the yield of recombinant proteins by decreasing the rate of protein synthesis, such as slower cell growth, growth in minimal media, lower induction and incubation temperatures which may offer better conditions to proteins (Marco *et al.*, 2005). Under normal growth conditions, aggregates or inclusion bodies may accumulate rather than the desired soluble, active form of the protein. Therefore optimisation of the conditions may limit this accumulation.

Further studies must therefore be performed on the thioredoxin expression system, with particular reference to improving protein expression. This may be achieved by using different growth conditions, such as varying the growth or induction temperature between 25 and 37°C. This may also improve the solubility of the fusion proteins and consequently aid purification of the target His-tagged proteins. A further consideration may be trying different bacteria strains, such as strains were codon usage is optimised for expression of mammalian cells.

Differences in codon usage between mammalian and bacteria may lead to a variety of problems regarding gene expression. Bacterial strains such as the Rossetta host strains (Novagen) allow expression of eukaryotic genes containing seven codons; AUA, AGG, AGA, CUA, CCC, GGA, and CGG, which are rarely used in *E. coli.*

Following the results on Ov8 Exon 1 2.1kb, Ov9 and ORF65, further studies were initiated on a wider range of genes. Several of the unique open reading frames (ORFs) in the OvHV-2 genome demonstrate significant homology with cellular proteins, suggesting they have been acquired from host DNA during co-evolution of the virus and its host (Coulter *et al.*, 2001). Included are genes encoding a homologue of a cellular interleukin (IL10 homologue Ov2.5), regulation of apoptosis (bcl2 homologues Ov4.5 and Ov9) and a virion glycoprotein (Ov7). The four genes selected as candidates for analysis were amplified by PCR and cloned into the bacterial expression vector pTrxFus so that they could be analysed to determine

whether they may be detected by antibodies in sera from MCF-infected animals. The thioredoxin vector system, pTrxFus, was used for this because it gave the best expression results among the systems tested. Based on results from this study, the solubility of the OvHV-2 proteins remained an issue, with the majority of proteins being insoluble as expressed from pTrxFus vector. Subsequent purification of the soluble proteins identified proved ineffective by His-tag specific methods.

In this system, pTrxFus-Ov2.5 and pTxFus-Ov7 were expressed but insoluble, while no expression was detected for pTrxFus-Ov3.5 and pTxFus-Ov8.5. Expression in eukaryotic cells has been recently demonstrated for Ov2.5, which was shown to be both secreted and functional (D. Haig, J., Stewart, unpublished data). HEK293T cells were transiently transfected with a vector (pVR1255) encoding Ov2.5 with C-terminal haemagglutin (HA) epitope tags and harvested after 48 hours. pVR1255 is a gene therapy vector which contains a CMV promoter and offers no drug resistance for selection in mammalian cells (Manthorpe *et al.*, 1993; Norman *et al., 1997*). Modifications to this vector included the addition of multiple cloning sites and a triple HA-tag for purification and detection of the cloned protein (G.Russell, unpublished data).

The tagged protein was expressed at high level and could be purified from the culture medium. However the Ov2.5 protein was not recognised by anti-MCF sera (data not shown), indicating that Ov2.5 was not an antigenic target in infected susceptible animals. Possible reasons for this result might be that Ov2.5 may not be expressed in animals affected with MCF. Alternatively, it may only be expressed at low levels. As Ov2.5 acts as a cytokine it is only required in small amounts, unlike the large amounts required for structural proteins such as ORF65 or glycoprotein Ov7, therefore there may not have been sufficient levels expressed to induce an immune response.

This work has shown that pTrxFus-ORF65 and pET22b-Ov8 Exon 1 2.1kb can be expressed, although further work is required to resolve issues concerning solubility and purification. The OvHV-2 genes studied may prove useful in the development of an ovine herpesvirus-2 recombinant protein based diagnostic ELISA. Alternative options to improve expression, solubility and purification issues may be achieved by using several systems such as yeast, baculovirus or mammalian expression systems. One aspect which may inhibit solubility is the presence of

inclusion bodies in bacteria, therefore protein refolding may also have to be considered for the insoluble proteins.

An additional option that could take advantage of the currently available bacterial constructs and cloned genes may be the use of novel systems for the expression of genes, such as the *in vitro* Rapid Translation System (RTS). RTS is a high-yield cell-free protein expression system, based on the use of T7 RNA polymerase and T7 promoter vectors to limit expression to genes of interest. This *in vitro* transcription-translation system avoids culture and induction problems and provides a rapid alternative for expression of candidate genes. As the system is cell-free, the background interference caused by bacterial proteins may also be reduced.

Chapter Four

In Vitro Expression Studies: The Rapid Translation System

4.1 Introduction

Previous studies for the expression of candidate antigens were performed using bacterial expression systems as they offer rapid expression of selected antigens in a form which may be easily purified, through the addition of a His-epitope tag.

As it was not possible to express and purify these candidate antigens an alternative expression system was sought. The *in vitro* Rapid Translation System (RTS) was selected. The use of *in vitro* translation systems can have advantages over *in vivo* gene expression when the product is insoluble or forms inclusion bodies, when the protein undergoes rapid proteolytic degradation by intracellular proteases or when the over-expressed product is toxic to the host cell (RTS application manual for cell-free protein expression, Roche). RTS is a high-yield cell-free protein expression system, based on the use of the T7 promoter and T7 RNA polymerase to limit expression to genes of interest.

This *in vitro* transcription-translation system avoids culture and induction problems and provides a rapid alternative for the expression of candidate genes. As the system is cell-free, the background interference caused by bacterial proteins may also be limited. The RTS system employs an enhanced *E. coli* lysate to perform coupled *in vitro* reactions, where a continuous source of energy substrates, nucleotides and amino acids combined with the removal of by-products (Figure 4.1) ensures high yield protein production of up to several milligrams (Betton, 2003; Rogé & Betton, 2005).

E. coli cell-free systems consist of a crude extract that is rich in endogenous mRNA. The extract is incubated during preparation so that this endogenous mRNA is translated and subsequently degraded. As the levels of endogenous mRNA in the prepared lysate are low, the exogenous product is easily identified. In comparison with eukaryotic systems, the *E. coli* extract has a relatively simple translational apparatus with less complicated control at the initiation level, allowing this system to be very efficient in protein synthesis. Bacterial extracts are often unsuitable for translation of RNA, because exogenous RNA is rapidly degraded by endogenous nucleases. There are some viral mRNAs (TMV, STNV, and MS2) that translate efficiently, because they are somewhat resistant to nuclease activity and contain stable secondary structure. However, *E. coli* extracts are ideal for coupled transcription-translation from DNA templates.

In standard translation reactions, purified RNA is used as a template for translation. "Linked" and "coupled" systems, on the other hand, use DNA as a

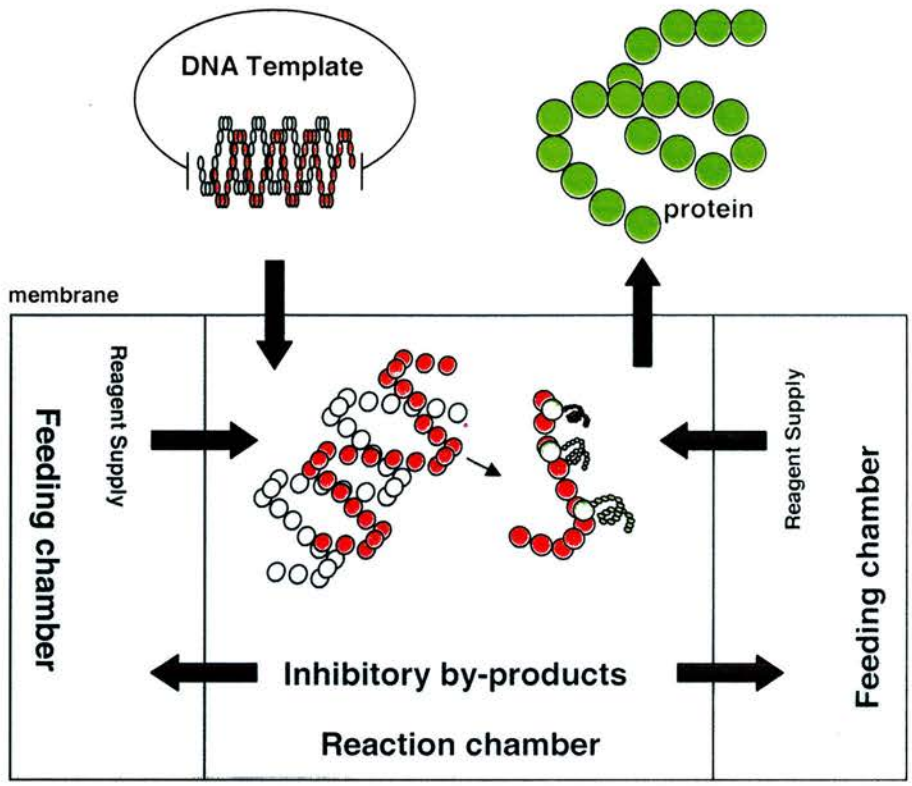


Figure 4.1: Illustration of RTS protein expression. Diagram modified from the Roche RTS application manual. The continuous exchange cell-free system (CECF) prevents the accumulation of inhibitory reaction by-products and prevents the exhaustion of reaction substrates. The columns semi-permeable membrane enables a continuous supply of substrates, while removing inhibitory products from the reaction chamber.

template. RNA is transcribed from the DNA and subsequently translated without any purification. Such systems typically combine a prokaryotic phage RNA polymerase and promoter (T7, T3, or SP6) with eukaryotic or prokaryotic extracts to synthesize proteins from exogenous DNA templates. DNA templates for transcription-translation reactions may be cloned into plasmid vectors or generated by PCR

The candidate genes are cloned into an RTS-specific plasmid vector, one of the pIVEX plasmid vectors. The pIVEX expression vectors are designed for high-level protein expression and are optimized for expression using the RTS approach, with T7 RNA polymerase and prokaryotic cell lysates. The vectors include the T7 gene translation enhancer, multiple cloning sites and a T7 transcription terminator.

The pIVEX family of vectors allows gene fusion to a variety of epitope tags for protein isolation (Rogé & Betton, 2005). The pIVEX2.3 and pIVEX2.4a vectors were selected as they contained C- or N-terminal His-tags respectively (Figures 4.2 and 4.3). Cloning into the NcoI sites in these vectors places the gene between the target peptide and the HisTag and so follows the cloning strategy designed for other vectors.

This chapter describes the expression of OvHV-2 genes using the *in vitro* Rapid Translation System. Previous study of OvHV-2 genes in bacterial expression systems revealed that OvHV-2 ORF65 (major capsid protein) and OvHV-2 Ov8 Exon 1: 2.1kb and 0.5kb (virion glycoprotein) could be expressed, although problems regarding solubility and purification in the bacterial lysates remained an issue. Although expression of Ov8.5 was not detected in the previous study using the thioredoxin fusion system, Ov8.5 was included in this *in vitro* study due to its unique nature. The expression of these OvHV-2 genes, cloned into *in vitro* expression vectors pIVEX2.3 or pIVEX2.4a, was demonstrated using western blotting with epitope-tag-specific antibodies and anti-MCF antibodies in serum.

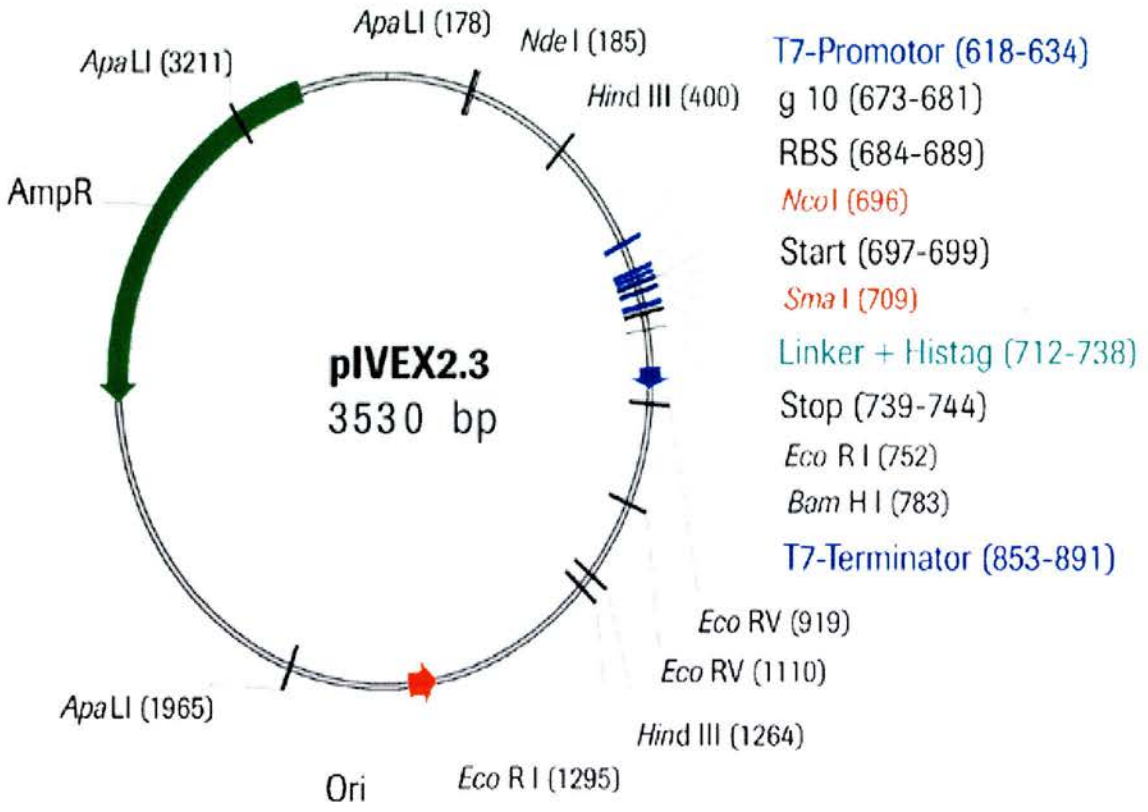


Figure 4.2: The pIVEX 2.3 expression vector (adapted from Roche). This vector carries regulatory elements required for in vitro expression with T7 RNA polymerase and prokaryotic cell lysates. pIVEX2.3 contains multiple cloning sites and an C-terminal polyhistidine (6xHis) tag for purification with nickel-chelating resin.

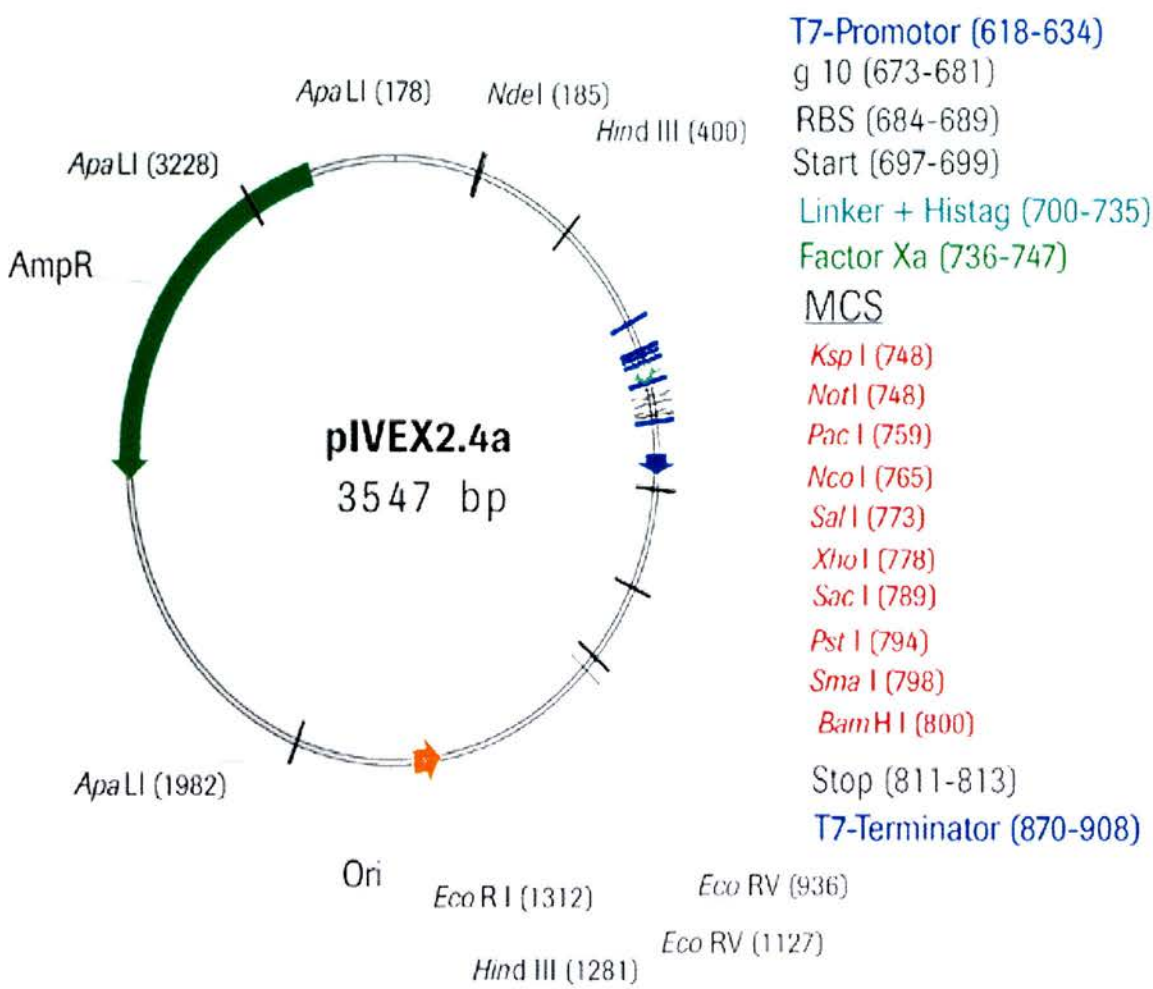


Figure 4.3: The pIVEX 2.4a expression vector (adapted from Roche). This vector carries regulatory elements required for in vitro expression with T7 RNA polymerase and prokaryotic cell lysates. pIVEX2.4a contains multiple cloning sites and an N-terminal polyhistidine (6xHis) tag for purification with nickel-chelating resin.

4.2. Material and Methods

4.2.1. Cloning into pIVEX Vectors

For this work, two vectors were used; pIVEX 2.3 (Figure 4.2) and pIVEX 2.4a (Figure 4.3). The name pIVEX represents the system (*In Vitro* EXpression); while the first number represents the basic vector, and the second number indicates the position of the His-tag within the vector (e.g. pIVEX 2.3 has a C-terminal HisTag while pIVEX 2.4a has an N-terminal tag). The letter "a" on the pIVEX2.4 vector represents a difference in open reading frame.

4.2.2. Digestion of pIVEX vectors

All cloned fragments had NcoI sites at both ends which could be cloned in-frame into pIVEX2.3 or pIVEX2.4a. Digestion of the pIVEX vector with the restriction enzyme NcoI was followed by de-phosphorylation to prevent self-ligation of the vector. Linearized vector DNA was purified by gel extraction. The target gene inserts Ov8 Exon 1: 2.1kb, Ov8 Exon 1: 0.5kb, Ov8.5 and ORF65 were excised from existing vectors by restriction digestion with restriction enzyme NcoI, as the genes were already flanked by NcoI sites.

4.2.3. Ligations using the pIVEX vectors (pIVEX 2.3 and 2.4a)

Ligation reactions contained 5µl 2x rapid ligation buffer, 1µl (50ng) vector, 1µl T4 DNA ligase (3 Weiss units/µl) and up to 3µl purified insert DNA, in a reaction volume of 10µl. Negative control reactions were included to check for self-ligation of the vector. Ligation reactions were incubated at room temperature for 1 hour then overnight at 4°C.

4.2.4. *In Vitro* Protein Expression

The target genes, once cloned into the pIVEX plasmid, were transformed into *E. coli* strain JM109 to amplify the expression plasmid. Glycerol stock cultures of *E. coli* carrying plasmids encoding fusion proteins were inoculated onto LB Medium agar plates with ampicillin (100µg/ml) and incubated overnight at 37°C. Single colonies were used to inoculate 10ml L-broth (Appendix 1l) containing 100µg/mg ampicillin and the cultures were incubated at 37°C (225rpm) overnight. The cultures were harvested by centrifugation at 3000 x g for 10 minutes, and plasmid DNA was purified using a QIAprep spin miniprep kit (method described in section 2.4.6.2).

RTS reaction reagents were prepared as stated by the manufacturer (RTS 500 Instruction manual, version 2). Between 10-15μg of DNA (≤50μl) was added to the reaction solution and loaded into the 1ml RTS reaction chamber. After 24 hours (30°C/120rpm), the reaction solution (1ml) was clarified at 1300 x g for 10 minutes to determine solubility of the RTS extract

4.2.5. Affinity Purification under Native and Denaturing Conditions

Using a Ni-NTA affinity resin purification column under native conditions, a total of 500μl of RTS extract (diluted 1:5 in binding buffer) was injected onto the column using an Akta FPLC system (with in-line analysis at 280nm).

The column was washed with distilled water and binding buffer (flow rate 1ml/min) prior to injecting the sample, to ensure no contaminants were present. The diluted sample was then injected onto the column, and the column was washed with binding buffer (50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole, pH8.0) until a constant baseline was reached. Elution buffer (50mM NaH_2PO_4, 300mM NaCl, 500mM imidazole, pH8.0) was then applied to the column and 1ml fractions were collected. Fractions corresponding to protein peaks on the FPLC system trace were collected and analysed by staining and western blotting using HisProbe-HRP antibody.

As purification of the fusion proteins was not achieved using Ni-NTA affinity purification under native conditions, purification under denaturing conditions (100mM NaH_2PO_4, 10mM Tris·Cl, 8M urea, pH8.0) was also performed.

4.2.6. NHS HisTrap purification

NHS HisTrap columns are commonly used for the purification of histidine-tagged recombinant proteins using immobilized metal ion (Ni) affinity chromatography. This purification column was used as an alternative purification method utilizing the HisTag. The RTS extract (100μl) was adjusted to the composition and pH of the binding buffer by diluting in binding buffer (20mM NaH_2PO_4, 0.5M NaCl, 20mM imidazole) to a final volume of 500μl.

The column was washed with distilled water and binding buffer (flow rate of 1ml/min) prior to injecting the sample, to ensure no contaminants were present in the column. The diluted sample was then injected onto the column, and the column was washed with binding buffer until a constant baseline was reached. Elution buffer (20mM sodium phosphate, 0.5NaCl, 500mM imidazole pH7.4) was then applied to

the column and fractions which corresponded to protein peaks on the FPLC system trace were collected and analysed by staining and western blotting using HisProbe-HRP antibody.

4.3. Results

4.3.1. pIVEX2.3-Ov8.5

The unique NcoI restriction site in the pIVEX vector was used for cloning and resulting colonies were subject to restriction analysis to define insert orientation, using the restriction enzyme PciI. One colony showed the correct orientation (Figure 4.4).

Fifteen micrograms of pIVEX2.3-Ov8.5 plasmid DNA was added to 1ml of RTS working solution. The RTS reaction was run at 30°C for 24 hours, stirring at 120rpm to ensure distribution and prevent membrane congestion.

A sample of the Ov8.5 RTS extract was mixed with 2x SDS loading buffer and 20µl was electrophoresed on a 14% NuPAGE tris-glycine polyacrylamide gel. No negative control was used as there was no RTS reaction reagents prepared in the absence of plasmid DNA. The stained gel showed no obvious bands for pIVEX2.3-Ov8.5, suggesting the protein was not over-expressed (Figure 4.5a). A duplicate gel was analysed by western blot using an anti-HisTag monoclonal antibody, to determine the presence of recombinant proteins. The pIVEX2.3-Ov8.5 expressed a clear polypeptide at 36kDa, which represents the Ov8.5 protein, with an expected size of 40kDa. Further bands were identified at approximately 12, 20 and 30kDa (Figure 4.5b), which suggests that there may be a level of protein degradation.

Bacterial expression systems proved unsuitable for the expression of OvHV-2 Ov8.5 (described in section 3.3.4), the cell-free *in vitro* system (RTS), however proved effective.

4.3.1.2. pIVEX-2.3 Ov8.5 affinity chromatography

In order to eliminate potential non-specific bands and clearly identify the Ov8.5 fusion protein, the Ov8.5 RTS extract was purified under native conditions using standard affinity (Ni-NTA) chromatography. Twenty microlitres of unbound lysate, along with the resin-bound eluant, were subjected to electrophoresis and stained with Sypro ruby stain (Figure 4.6a). The unbound and wash fractions were heavily stained like the RTS extract. A band at 38kDa, the expected size of the Ov8.5 protein, was additionally observed in the unbound and wash fractions but was not visible on the resin, suggesting that either most of the protein present remained unbound or was non-specific. Bands were faintly visible in the eluted extract at 25, 28 and 49kDa which did not appear to represent highly expressed bands in the crude extract. The presence of these expressed proteins could be due the occurrence of non-

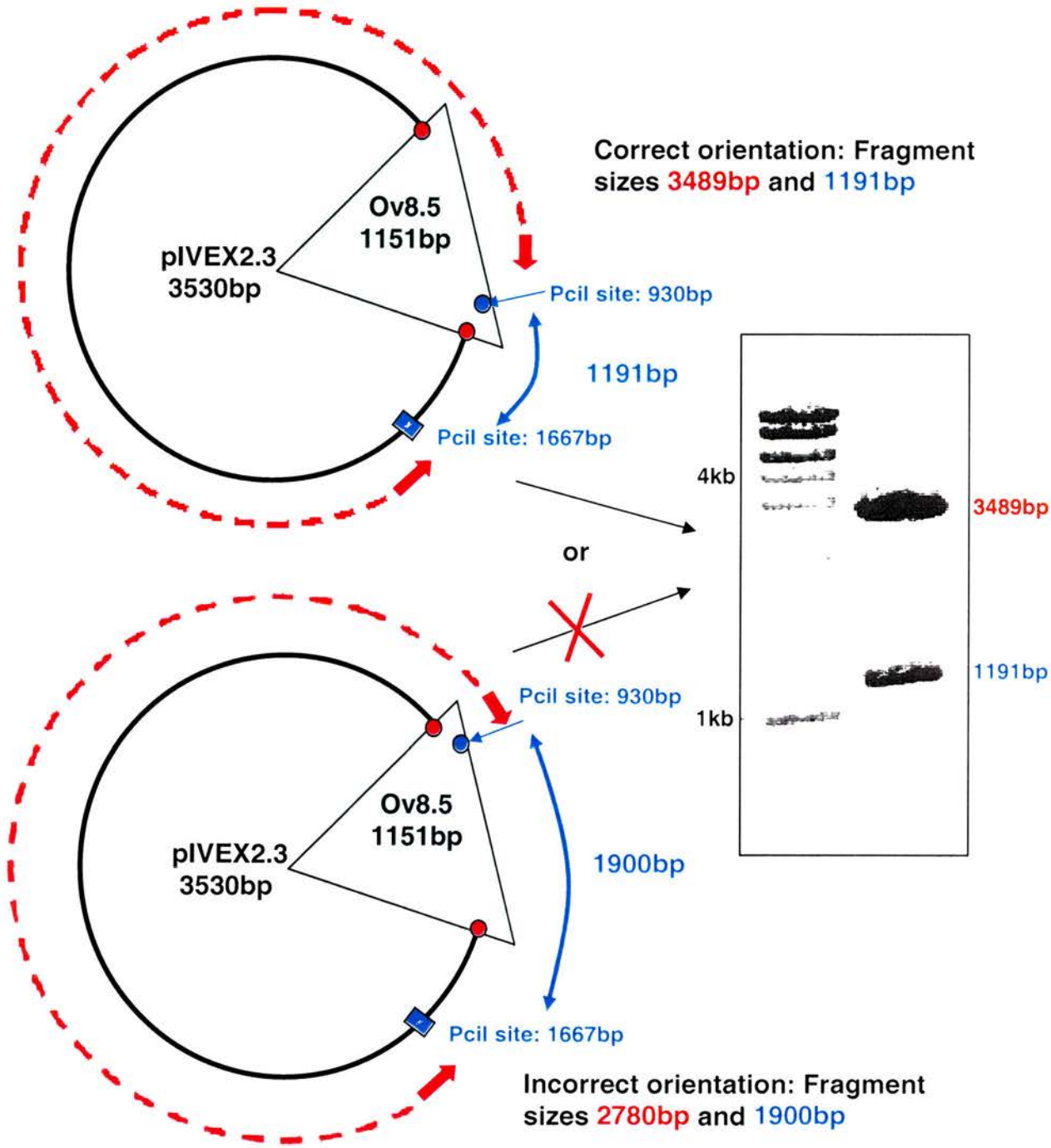


Figure 4.4: pIVEX2.3 OvHV-2 Ov8.5.

Orientation determined by restriction digest reaction with enzyme PciI ( ). Unique site in pIVEX2.3 vector at 1667bp (3530bp), and 930bp in the insert (1151bp insert). Result in two bands at 3489kb and 1191kb. ● represents NcoI restriction sites, Hyperladder I used as DNA marker, Bioline.

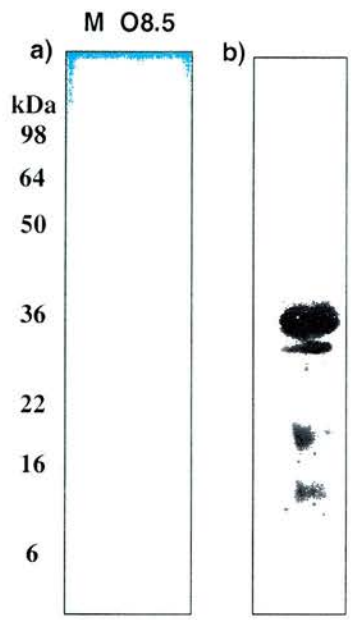


Figure 4.5: pIVEX2.3 Ov8.5 RTS extract detected by staining and chemiluminescence western blot. Twenty microlitres of Ov8.5 RTS extract separated by electrophoresed on a 14% tris-glycine polyacrylamide gel was analysed by a) SimplyBlue SafeStain and b) western blotting using anti-HisTag antibody as a primary antibody followed by anti-mouse HRP conjugate. The bound conjugate was detected by chemiluminescence using the Pierce Supersignal ELISA femto HRP substrate. Negative control not shown.

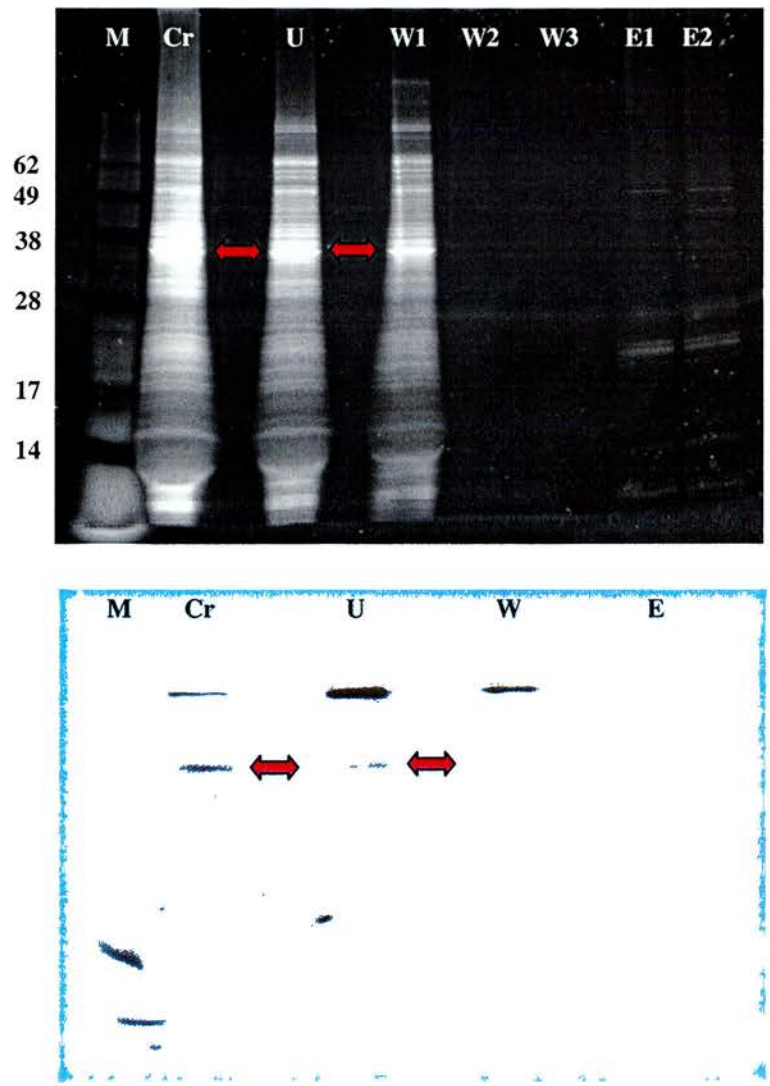


Figure 4.6: pIVEX2.3 Ov8.5 Ni-NTA affinity purification under native conditions. Twenty microlitres of the purified eluent (E) was fractionated on a 14% tris-glycine polyacrylamide gel alongside the crude lysate (Cr), unbound material (U) and washing stages 1-3 (W). The protein lysate was analysed by either staining with Sypro Ruby (a) or western blotting (b) using anti-HisTag antibody as a primary antibody followed by anti-mouse HRP conjugate. The bound conjugate was detected by chemiluminescence using the Pierce Supersignal ELISA femto HRP substrate. No control was used.

specific histidine binding to *E. coli* proteins which may interfere with the binding of the Ov8.5 fusion protein.

To confirm the presence of His-tagged proteins a western blot, using anti-HisTag antibody, was performed on samples from the purification procedure (Figure 4.6b). Similar to the stained gel, distinct His-tagged bands were observed in the RTS extract, unbound and wash fractions at approximately 38, 50 and 60kDa. The 50 and 60kDa proteins are most likely to be *E. coli* products while the 38kDa may be the Ov8.5 protein. No bands were observed in the SDS-treated pellet. This suggests that the Ov8.5 protein did not bind to the NTA column and possibly reflects the relative expression level of the Ov8.5 protein. Ni-NTA affinity purification was performed under denaturing conditions and showed distinct His-tagged bands in only the whole cell lysate, at approximately 28, 38, 50 and 60kDa (Figure 4.7). A distinct band at 38kDa was also detected in the wash fractions in the resin-bound eluant. This indicates that there is either a relatively low level of Ov8.5 protein expression or the Ni-NTA column resin binds this His-tagged protein poorly.

In order to determine whether the Ov8.5 protein remained in the fall-through fraction, a second affinity purification method was performed using 5-fold more material. The pre-packaged NHS-HisTrap column was loaded with Ov8.5 RTS extract (2.5ml), diluted 1:5 in binding buffer (20mM NaH_2PO_4, 0.5M NaCl, 20mM Imidazole). Twenty-five (1ml) fractions were collected and analysed, and fractions corresponding to protein peaks on the FPLC system UV trace were analysed by staining and western blotting using HisProbe-HRP antibody (Figure 4.8). Stained gels showed detectable protein bands in only peak fractions 12, 13 and 14, with apparent molecular weights ranging from 50 to 148kDa. Fractions 12 and 13 correspond to the first and highest protein peak from the fractionation and showed strong bands at 50 to 148kDa (Figure 4.8a). Fraction 14 also showed two major protein bands at 55 and 60kDa. The expected size of the Ov8.5 protein is 40kDa. A western blot, using an anti-HisTag antibody, of the same fractions showed that His-tagged proteins eluted in the first protein peak with apparent molecular weights ranging between 50 to 148kDa (Figure 4.8b). The second protein peak (Fraction 15-16) showed no protein expression by staining, however a band at approximately 36kDa was observed by western blotting. This 36kDa band could represent the Ov8.5 protein, but as no band was detected by SimplyBlue stain, it was assumed that the level of Ov8.5 protein expression was too low for further analysis.

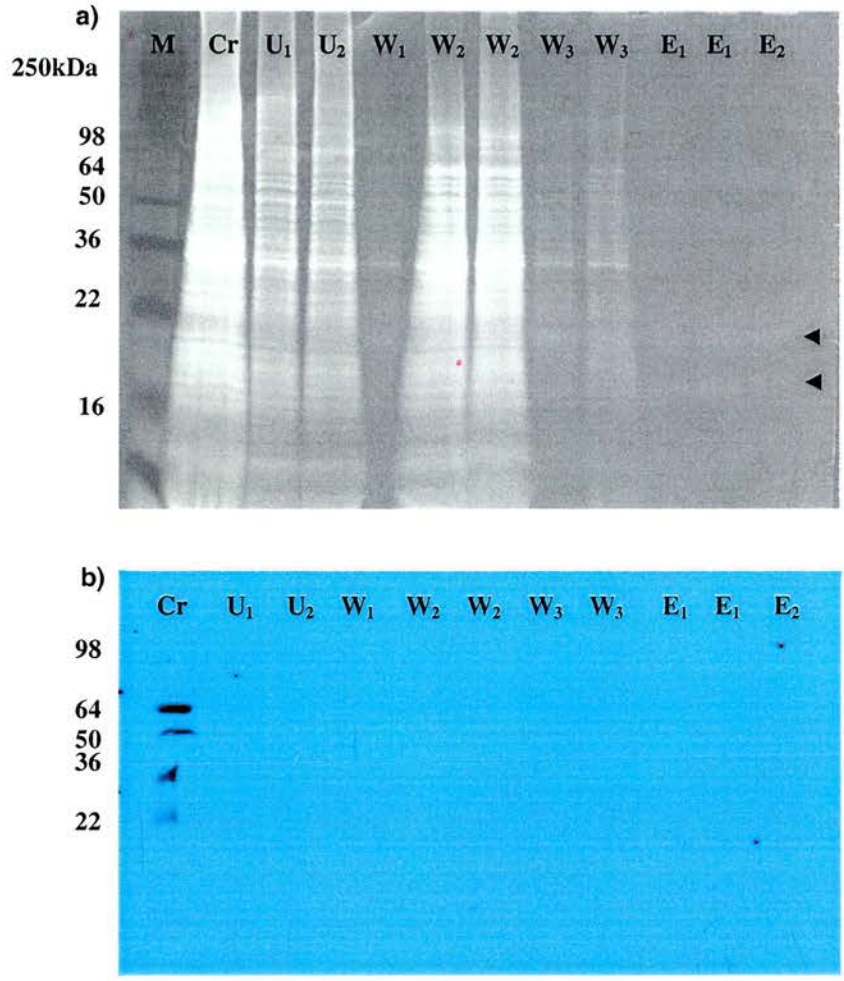


Figure 4.7: pIVEX2.3 Ov8.5 Ni-NTA affinity purification under denaturing condition. Twenty microlitres of the RTS extract (E) was fractionated on a 14% tris-glycine polyacrylamide gel alongside the crude lysate (Cr), unbound material (U) and washing stages 1-3 (W). The protein lysate was analysed by either staining with SimplyBlue SafeStain (a) or western blotting (b) using anti-HisTag antibody as a primary antibody followed by anti-mouse HRP conjugate. The bound conjugate was detected by chemiluminescence using the Pierce Supersignal ELISA femto HRP substrate.

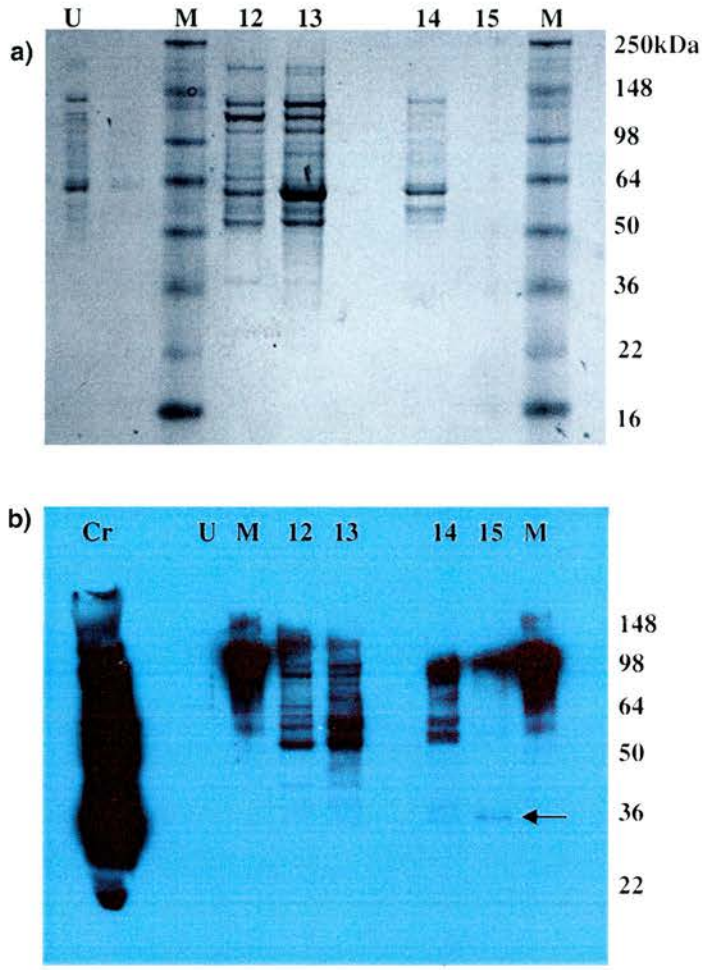


Figure 4.8: Purification of pIVEX2.3 Ov8.5 under denaturing conditions. pIVEX2.3-Ov8.5 extract was fractionated, on a 12% bis-tris polyacrylamide gel, alongside the unbound material (U) and crude lysate present as a control (Cr). The fractions were analysed by either staining with SimplyBlue Safestain (a) or western blotting using HisProbe-HRP antibody (b). The bound conjugate was detected by chemiluminescence using the Pierce Supersignal West Dura HRP substrate. Arrow indicates expected size of pIVEX2.3 Ov8.5 gene product.

4.3.2. pIVEX2.3-ORF65

The restriction enzyme BstXI was used to analyse the insert orientation of pIVEX2.3-ORF65 and a colony showing the correct orientation was selected (Figure 4.9). Fifteen micrograms of DNA was added to the RTS working solution and incubated (30°C/24hours). The reaction solution was removed and as there was precipitate visible, the extract was centrifuged at 1300 x g for 10 minutes to determine solubility of the extract. The soluble and insoluble phases were collected and analysed by electrophoresis and western blotting to determine whether any recombinant protein was present in the soluble phase. Using an anti-HisTag monoclonal antibody, no bands were shown in the insoluble phase suggesting that the ORF65 protein was soluble (data not shown).

The soluble fraction was analysed by staining and western blot, using a monoclonal HisTag antibody to detect the presence of the ORF65 fusion protein. MCF-positive and -negative antisera were also tested to determine whether the ORF65 protein is antigenically reactive to MCF serum (Figure 4.10b, c). Using the HisTag monoclonal antibody, the crude extract contained many reacting bands between 28 and 98kDa (expected size 38kDa). Both the MCF-positive and negative antiserum identified bands ranging between 35 and 64kDa, although no distinct protein band was shown. This could indicate that there was a high level of histidine binding in the crude lysate, possibly due to *E. coli* proteins present in the RTS reaction mix. Therefore, to remove any non-specific bands and to purify the ORF65 protein, the crude lysate was fractionated by Ni-NTA affinity purification under native conditions.

4.3.2.1. pIVEX-2.3-ORF65 affinity purification

Using the Atka FPLC system, twenty-eight 1ml eluted fractions were collected from the Ni-NTA column. As fractions corresponding to protein peaks on the FPLC system trace were difficult to identify, due to the presence of imidazole, the first four fractions of the elution were pooled and concentrated 5-fold using a 0.5ml microcon filter. The pooled fractions were analysed by staining and western blot, using HisProbe-HRP antibody, and showed no bands at the expected size for the ORF65 fusion protein. A 55kDa band was seen in the eluant which implies the presence of an *E. coli* product. It can therefore be assumed that either the ORF65 protein was not bound to the column or not eluted (Figure 4.11). A 45kDa band was also seen in the SDS-treated pellet, and may similarly indicate an *E. coli* product.

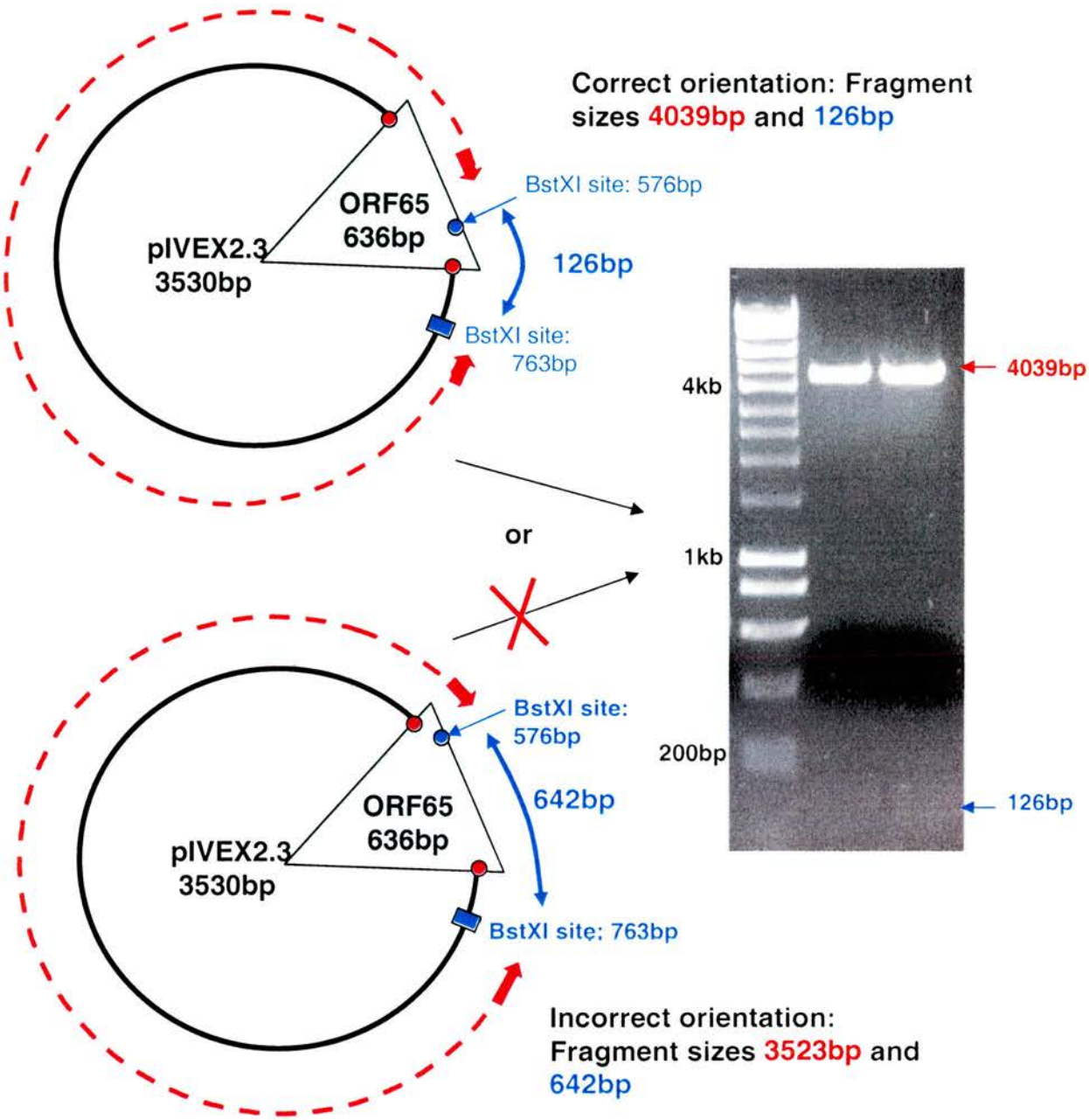


Figure 4.9: Orientation of pIVEX2.3 OvHV-2 ORF65.

Orientation determined by restriction digest reaction with enzyme BstXI ( ). Unique site in pIVEX2.3 vector at 763bp (3530bp), and 576bp in the ORF65 insert (636bp). The correct orientation would yield bands of 4039bp and 126bp, while the opposite orientation would yield bands of 3523bp and 642bp. represents NcoI restriction sites. Hyperladder I used as DNA marker, Bioline.

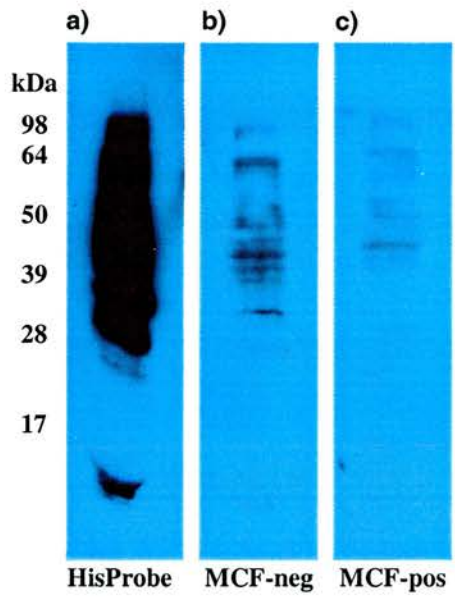


Figure 4.10: Chemiluminescent western blot of pIVEX2.3-ORF65 RTS extract.
Expression of the soluble pIVEX2.3-ORF65 extract was analysed by western blotting using (a) HisProbe-HRP, (b) MCF-negative antiserum and (c) MCF-positive antiserum, using a rabbit anti-bovine HRP conjugate. The bound conjugate was detected by chemiluminescence using the Pierce Supersignal West Dura HRP substrate.

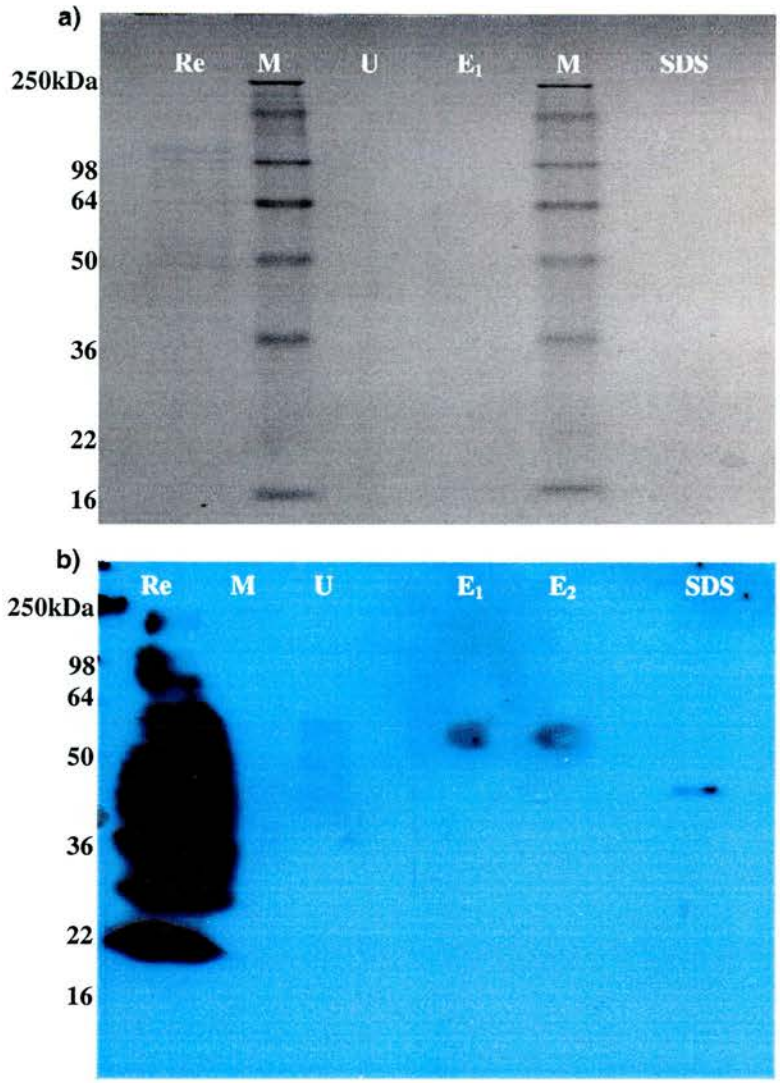


Figure 4.11: Electrophoretic analysis of affinity purified pIVEX2.3-ORF65. Twenty microlitres of pIVEX2.3-ORF65 crude extract (Re), the unbound fraction (U), eluent (E) and SDS-treated column resin (SDS) were loaded onto a 10% tris-glycine polyacrylamide gel and run at 100V for 1 hour. The samples were analysed by staining using SimplyBlue SafeStain (a) and western blotting using HisProbe-HRP antibody (b). The bound conjugate was detected by chemiluminescence using the Pierce Supersignal West Dura HRP substrate.

Similar to Ov8.5, purification under denaturing conditions using an NHS HisTrap column was performed. Twenty-five 1ml fractions were collected, using the Akta FPLC system, and fractions corresponding to the two protein peaks on the FPLC system trace were analysed by electrophoresis and western blot (Figure 4.12).

Using SimplyBlue staining several protein bands were seen in the first elution peak, corresponding to fractions 12 and 13, with apparent molecular weight between 30kDa and 148kDa. The second peak, corresponding to fraction 16, showed only a single, faint band at approximately 80kDa. By western blot, using an anti-HisTag antibody, a high level of His-positive protein expression in the crude lysate was shown. In the fractions from the first peak, protein was detected at 60 and 65kDa. No protein bands were detected in fractions from the second protein peak. Therefore, as no His-tagged protein was detected at the size expected for the ORF65 fusion protein, it was assumed that the level of protein expression was too low for detection by western blot.

Expression of the pIVEX2.3-ORF65 fusion protein indicated a soluble protein, though purification by native and denaturing conditions proved unsuccessful. This might suggest that the HisTag epitope tag was embedded in the protein and therefore not exposed to the column. To determine whether the tag was hidden, the pIVEX2.4a vector was employed, with the HisTag present on the N-terminus rather than the C-terminus.

4.3.3. pIVEX 2.4a-ORF65

The restriction enzyme PstI was used to analyse insert orientation of pIVEX2.4-ORF65, and a colony showing the correct orientation was selected (Figure 4.13) and 12μg of DNA was loaded into the RTS reaction chamber. After 24 hours (30°C/120rpm), the reaction solution was clarified at 1300 x g for 10 minutes to determine solubility of the RTS extract and, like the pIVEX2.3-ORF65 fusion protein, protein bands were found in the soluble fraction (Figure 4.14). Non-specific bands were detected by both the His-Tag monoclonal antibody (Figure 4.14a, b) and anti-MCF serum (Figure 4.14c, d) in both the soluble and insoluble fractions, suggesting the presence of non-specific protein binding by both antibodies and serum. A protein band at the size expected for ORF65 was not clearly detected by the anti-MCF sera, however a band at approximately 40kDa was seen in the soluble fraction, by the monoclonal HisTag antibody. This band may therefore represent ORF65.

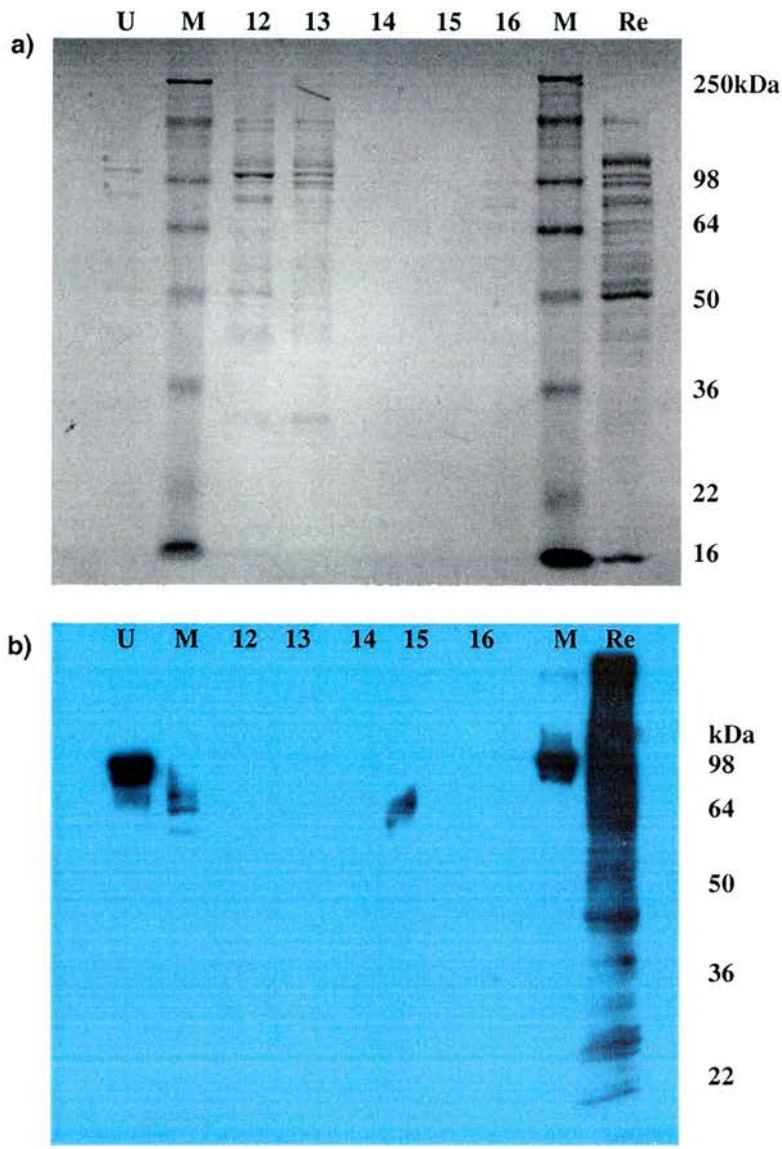


Figure 4.12: Electrophoretic analysis of pIVEX2.3-ORF65, purified under denaturing conditions. Fractions collected by HisTrap purification performed on an HPLC, were concentrated using a 0.5ml microcon filter and resuspended in equal volumes with 2x SDS sample buffer. Each fraction (15µl) was analysed by SDS-PAGE alongside the unbound material (U) and RTS extract (Re), present as controls. The electrophoresed fractions were analysed by SimplyBlue staining (a) and western blotting using HisProbe-HRP antibody (b). The bound conjugate was detected by chemiluminescence using the Pierce Supersignal West Dura HRP substrate.

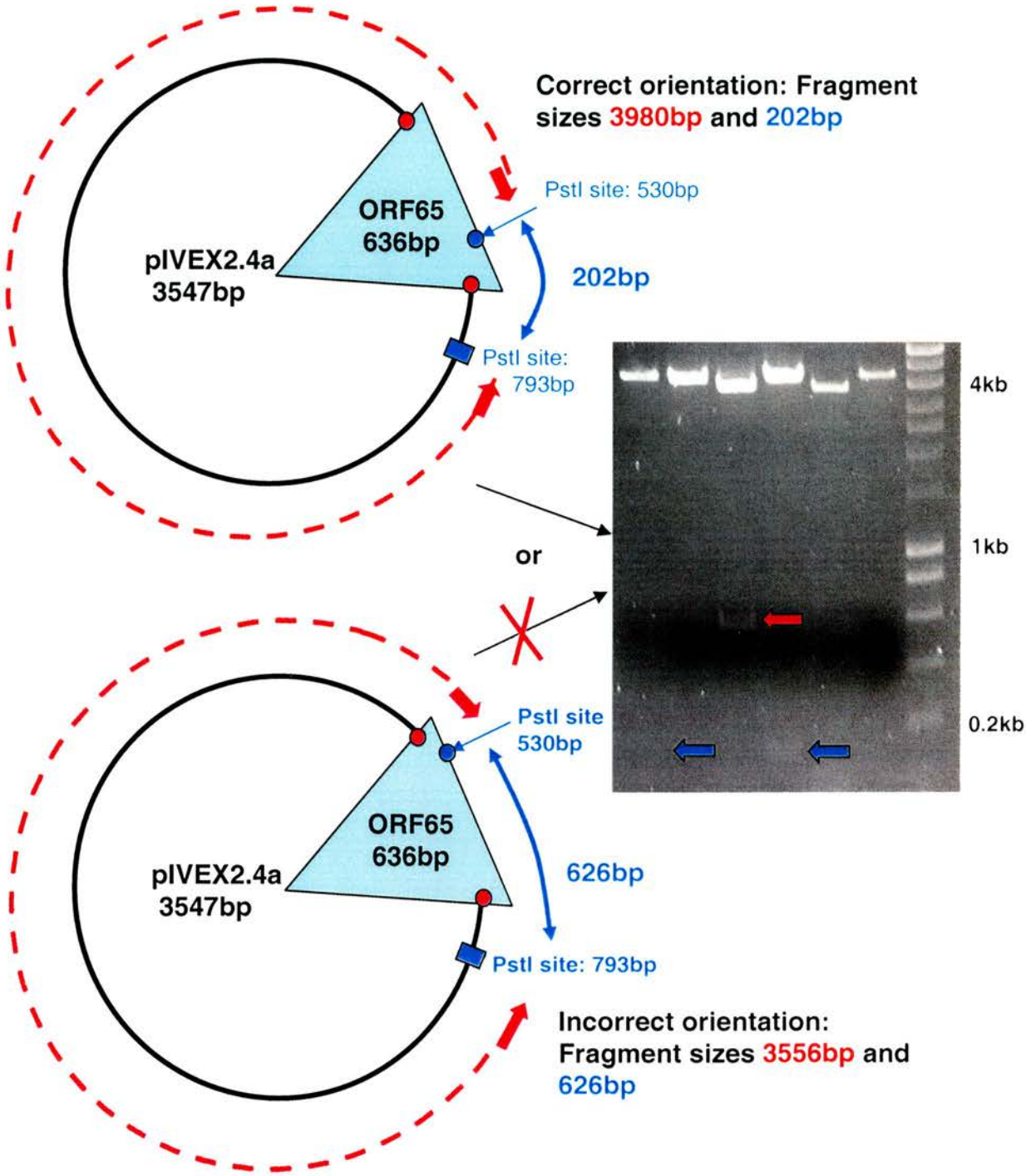


Figure 4.13: Orientation of pIVEX2.4a OvHV-2 ORF65.

Presence of insert demonstrated and orientation determined by restriction digest reaction with enzyme PstI (▬). Unique site in pIVEX2.4a vector at 793bp (3547bp), and 530bp in the ORF65 insert (636bp). Result in two bands at 3980bp and 202bp.

● represents NcoI restriction sites, Hyperladder I used as DNA marker, Bioline.

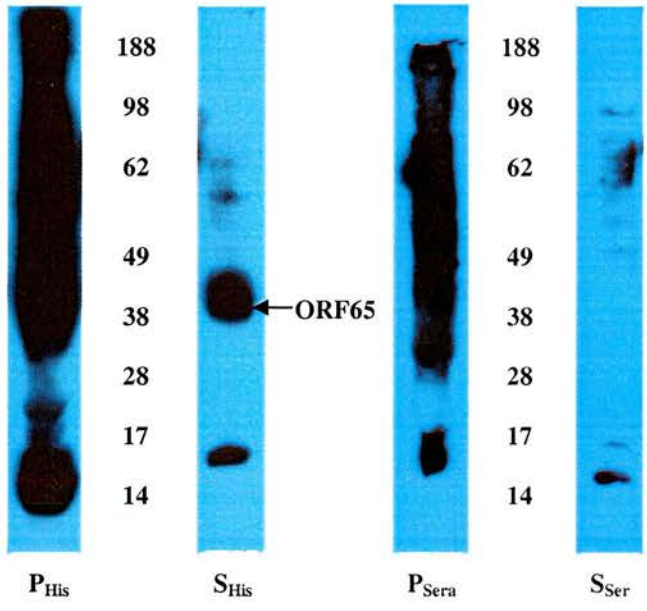


Figure 4.14: Chemiluminescent western blot of pIVEX2.4a-ORF65 RTS extract.
Expression of the insoluble (P) and soluble fractions (S) from the pIVEX2.4a-ORF65 extract were analysed by western blotting using (a,b) HisProbe-HRP, and (c, d) MCF-positive antiserum, using a rabbit anti-bovine HRP conjugate. The bound conjugate was detected by chemiluminescence using the Pierce Supersignal West Dura HRP substrate.

Purification was performed using an NHS HisTrap purification column under native conditions (500mM Imidazole).Twenty-five 1ml fractions were collected from the column, using the Atka FPLC system (Figure 4.15). Similar to pIVEX2.3-Ov8.5, fractions corresponding to protein peaks were difficult to analyse due to the presence of imidazole, therefore the first five fractions were concentrated 5-fold and analysed by staining (Figure 4.15a) and western blotting using an anti-HisTag monoclonal antibody (Figure 4.15b). No protein bands were found by staining. By western blot using an anti-HisTag monoclonal antibody, a faint band was seen at approximately 25kDa and a more strongly reactive band was seen at 97kDa. This result, similar to findings with the pIVEX2.3 vector, suggests the purification was ineffective in isolating the target protein, ORF65, possibly as a result of ineffective binding to the HisTrap column.

NHS HisTrap purification under denaturing conditions was performed. Twenty-five 1ml fractions were collected and fractions corresponding to protein peaks. Similar to pIVEX2.3-Ov8.5, fractions were analysed by staining (Figure 4.16a) and western blotting using an anti-HisTag monoclonal antibody (Figure 4.16b). Using SimplyBlue staining several protein bands were seen in the first elution peak, corresponding to fractions 15, 16 and 17, with apparent molecular weight between 39kDa and 97kDa.

By western blot, a high level of expression in the crude lysate was shown in the presence of urea, and bands were detected in fraction 17, at between 50 and 65kDa. A faint band was additionally detected, at 65kDa, in fraction 23 from the second protein peak. Therefore, as no His-tagged protein was detected at the size expected for the ORF65 fusion protein, it was assumed that the level of protein expression was too low for detection by western blot.

As the ORF65 protein could not be purified under native or denaturing conditions, it was assumed that it was the level of ORF65 expression and not its folding which was responsible for its lack of detection and therefore further analysis of this capsid protein was not continued. Non-specific bands were detected by both the His-Tag monoclonal antibody and anti-MCF serum. Although a band was detected with the monoclonal antibody at 40kDa, no protein band was seen at the size expected for ORF65 (38kDa) with anti-MCF sera. ORF65 was therefore not evaluated as a recombinant ELISA antigen.

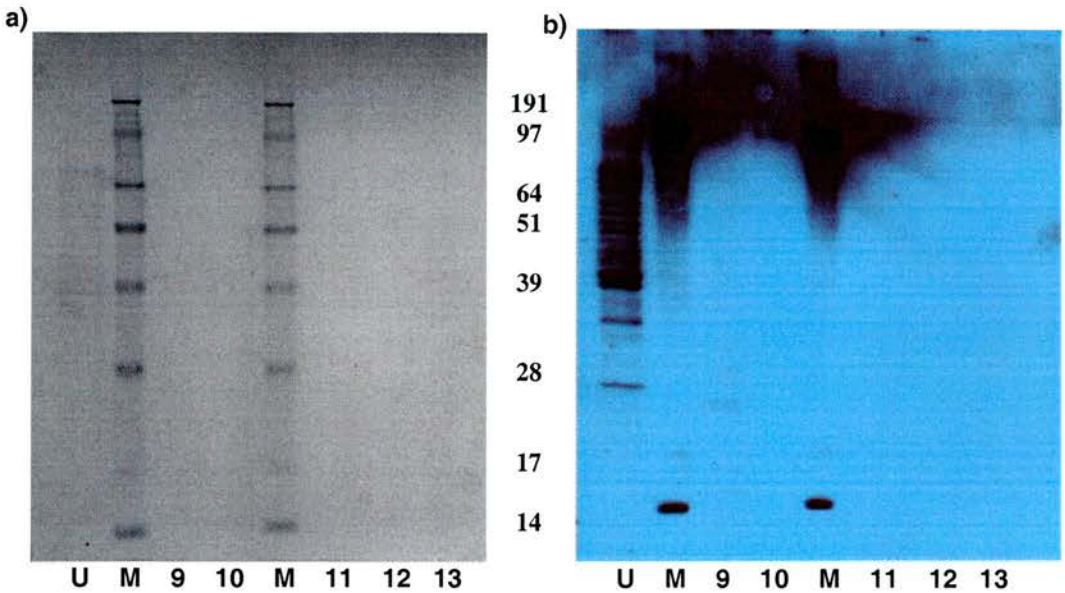


Figure 4.15: Electrophoretic analysis of HisTrap purified pIVEX2.4a-ORF65.
Fractions (1ml) were collected from a HisTrap purification of pIVEX2.4a-ORF65 by HPLC. Fractions showing peaks on the chromatogram (fractions 9-13) were concentrated in a 0.5ml micron filter and resuspended in equal volumes in sample loading buffer. Individual fractions (15µl) were loaded onto a 10% tris-glycine polyacrylamide gel and run at 100V for 1 hour, alongside the unbound material (U) present as a control. The fractions were analysed by a) staining with SimplyBlue SafeStain and b) western blotting using HisProbe-HRP antibody. The bound conjugate was detected by chemiluminescence using the Pierce Supersignal West Dura HRP substrate.

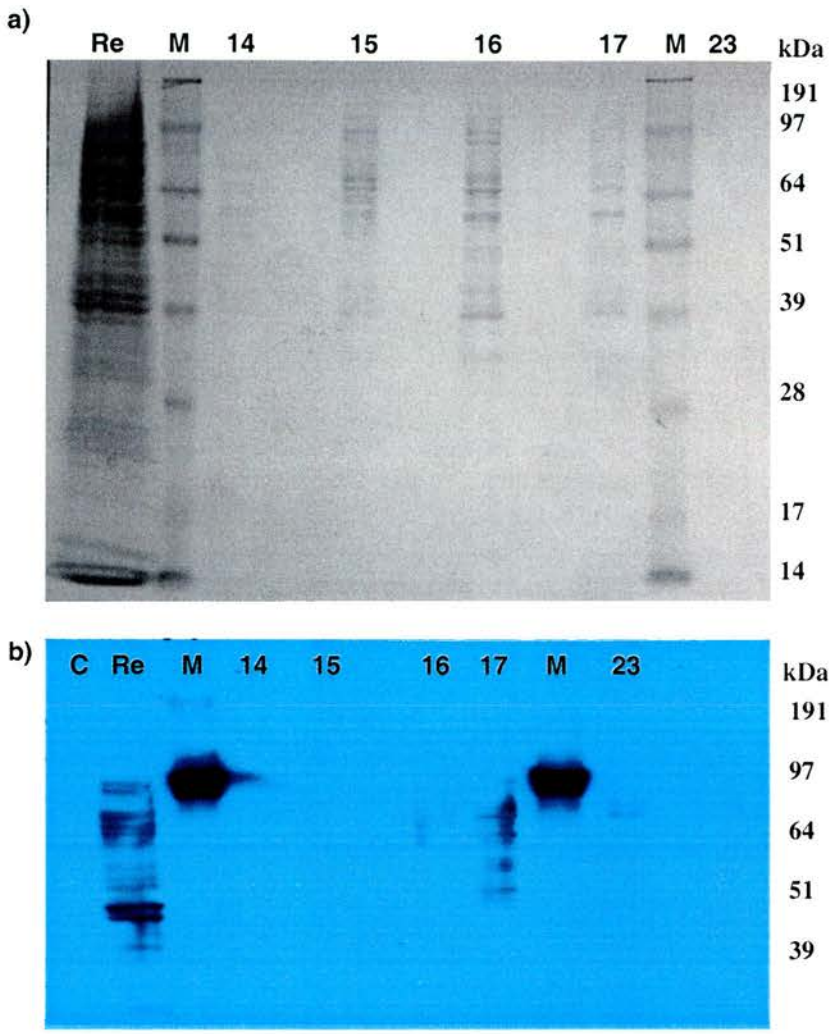


Figure 4.16: HisTrap purification of pIVEX2.4a-ORF65 under denaturing conditions. Fractions (14-23) were electrophoresed alongside the RTS extract (Re) present as a control. C represents a negative control. The fractions were analysed by a) staining with SimplyBlue SafeStain and b) western blotting using HisProbe-HRP antibody. The bound conjugate was detected by chemiluminescence using the Pierce Supersignal West Dura HRP substrate.

4.3.4. pIVEX2.3-Ov8 Exon 1 (2.1kb)

Although the pIVEX2.3-Ov8 Exon 1 2.1kb was shown to be insoluble in Chapter 3, an attempt to overcome solubility issues was performed. This was carried out before conclusions were made regarding the analysis of the Ov8 gene fragment. A clone of pIVEX2.3-Ov8 Exon 1 2.1kb (Figure 4.17) with correct orientation was selected and 12.3µg of DNA was added to the RTS working solution. The RTS reaction was run for 24 hours (30°C/ 120rpm). The reaction solution was removed and centrifuged at 1300 x g for 10 minutes to determine solubility of the expressed protein. The protein was almost totally insoluble (Figure 4.18).

The final annotation of the OvHV-2 genome indicated a shorter exon and therefore the original construct may contain coded intron and thus may be responsible for the expression and solubility problems shown in both bacterial and cell-free *in vitro* expression systems. A 0.5kb fragment of Ov8 exon 1 was therefore cloned and successfully expressed in the thioredoxin fusion expression system.

The insoluble Ov8 exon 1 2.1kb product was centrifuged at 1000 x g for 10 minutes, and the pellet was resuspended in either 2x SDS and incubated at 65°C for 5 minutes, or ELISA coating buffer (0.2M Na_2CO_3, 0.2M$NaHCO_3$, pH9.6). Samples were fractionated on a 14% tris-glycine gel and analysed by western blotting using either anti-HisTag monoclonal antibody or MCF-positive sera (Figure 4.19)

The Ov8 RTS product, diluted 10-fold, clearly shows a strong band at 90kDa, the size expected for Ov8 exon 1 2.1kb, and a faint band at 64kDa. Different detergents, SDS and 1% Nonylphenylpolyethylene glycol (NP40) were used to solubilise the Ov8 protein. Extraction of the insoluble pellet-material with SDS showed a high level of non-specific proteins ranging from 16 to 250kDa by both anti-HisTag monoclonal antibody and polyclonal MCF-positive sera. Washing the SDS-treated pellet in PBS appeared to remove the majority of non-specific bands, with the exception of a band at 64kDa.

Extraction with NP40, a detergent used in the production of ELISA antigen (described in section 5.1.1), apparently failed to solubilised any protein, using the HisTag monoclonal antibody, while the MCF-positive sera showed a faint band expressed at 64kDa, but not the size expected for Ov8 Exon 1 2.1kb (90kDa).

Expression studies using the bacterial expression systems pBAD and pET22b showed expression of the Ov8 Exon 1 2.1kb protein (described in chapter three). However, rather than the expected protein band, two bands were obtained,

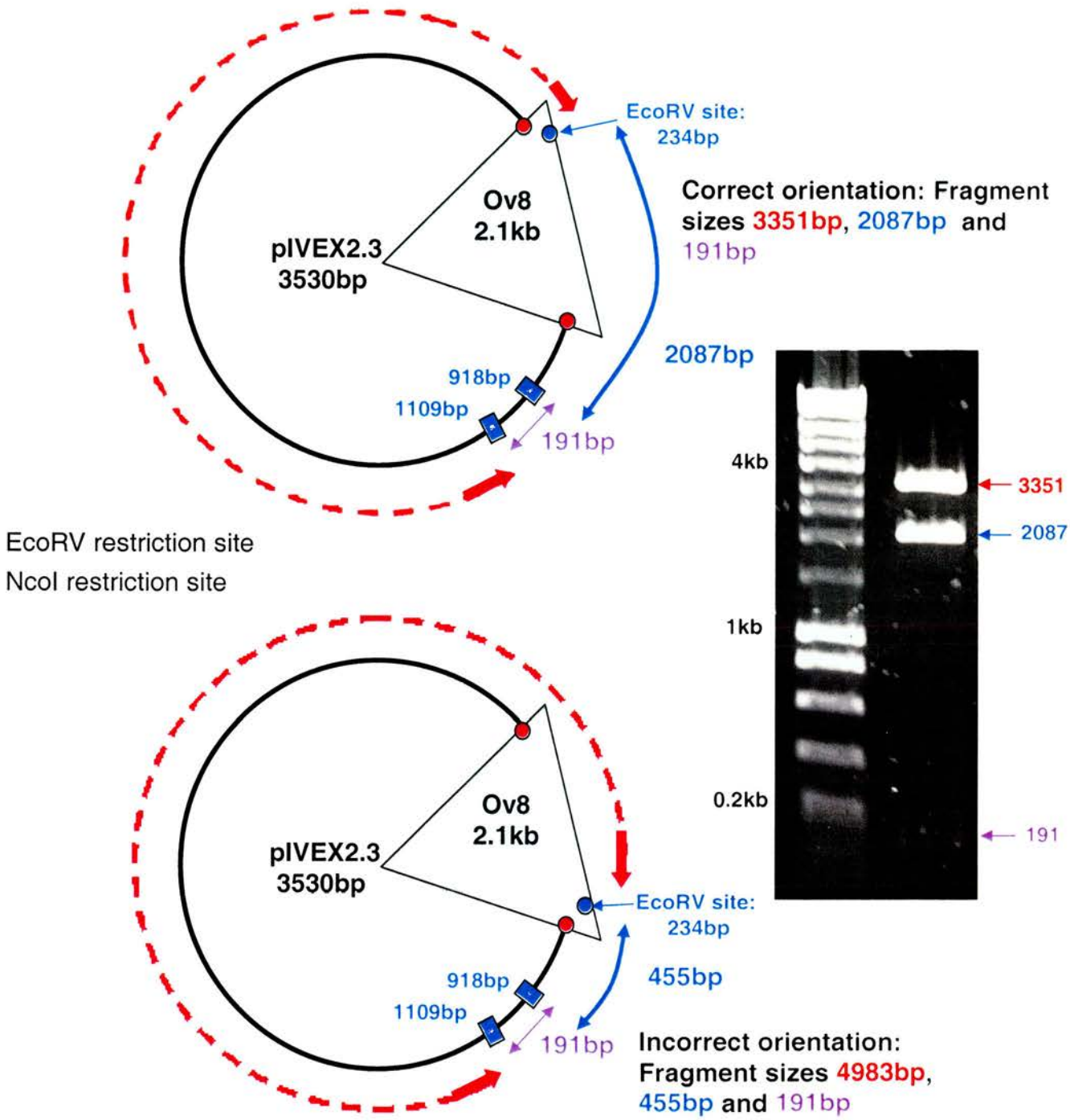


Figure 4.17: Orientation of pIVEX2.3-O8 (2kb).

Presence of insert demonstrated and orientation determined by restriction digest reaction with enzyme EcoRV (▬). Restriction sites in pIVEX2.3 vector at 918bp and 1.1kb (3547bp), and 234bp in the orf65 insert (2.1kb). Result in three bands at 3.1kb, 2kb and 191bp. ● represents NcoI restriction site, Hyperladder I used as DNA marker, Bioline.

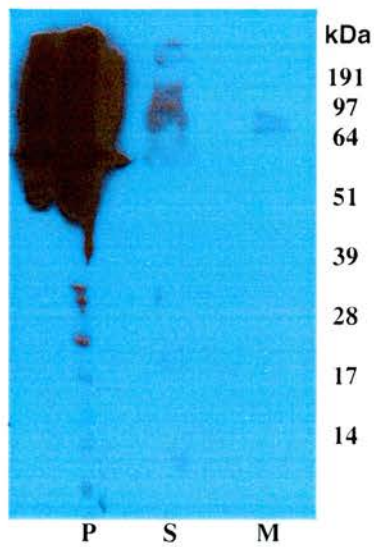


Figure 4.18: Chemiluminescent western blot of pIVEX2.3-Ov8 RTS extract (2kb). Expression of the electrophoresed soluble (S) and insoluble (P) fractions of the pIVEX2.3-Ov8 (2kb) extract analysed by western blotting using HisProbe-HRP, using a rabbit anti-bovine HRP conjugate. The bound conjugate was detected by chemiluminescence using the Pierce Supersignal West Dura HRP substrate. M represents protein marker SeeBlue Plus2 (Sigma), and shows a degree of reactivity against the chemiluminescence reagent.

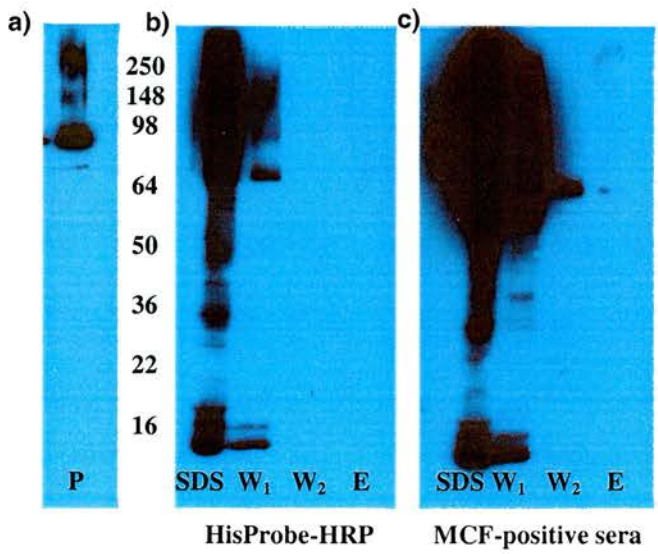


Figure 4.19: Chemiluminescent western blot of pIVEX2.3-Ov8 RTS extract (2kb).
a) Expression of the electrophoresed insoluble (P) fractions of the pIVEX2.3-Ov8 (2kb) extract treated with SDS buffer (SDS), washed in PBS (W_1 and W_2) and ELISA coating buffer (E). The gels were analysed by western blotting using b) HisProbe-HRP and c) MCF-positive sera, using a rabbit anti-bovine HRP conjugate. The bound conjugate was detected by chemiluminescence using the Pierce Supersignal West Dura HRP substrate.

suggesting the protein may have undergone proteolytic cleavage. Purification issues were also found for pET22b-Ov8, as the protein remained totally insoluble after mechanical lysis and even treatment with urea.

The final annotation of the OvHV-2 genome indicated Ov8 Exon 1 was only 1.2kb, and suggests the remaining 0.8kb in the construct used here may be responsible for the expression and solubility problems shown in both bacterial and cell-free *in vitro* expression systems. A 0.5kb fragment of Ov8 exon 1 was therefore cloned and successfully expressed in the thioredoxin fusion expression system. Purification of the pTrxFus-Ov8 protein under native and denaturing techniques

Purification of the pTrxFus-Ov8 protein under native and denaturing techniques appeared to be unsuccessful, as only *E. coli* superoxide dismutase was identified in proteomic analysis. The RTS cell-free system was therefore tested to possibly resolve purification issues.

4.3.5. pIVEX2.3-Ov8 Exon 1 (0.5kb)

A clone of the pIVEX2.3-Ov8 Exon 1 0.5kb (Figure 4.20) was selected and 14μg of DNA was added to the RTS working solution. After running the RTS reaction (24 hours at 30°C/120rpm), the reaction solution was removed and centrifuged at 1300 x g for 10 minutes to determine solubility of the expressed protein. The soluble and insoluble phases were collected and resuspended in 2x SDS loading buffer and analysed by electrophoresis and western blotting to determine whether any recombinant protein was present in the soluble phase (Figure 4.21).

Using an anti-HisTag monoclonal antibody, the majority of Ov8 expression was shown to be in the soluble phase, suggesting that the Ov8 exon 1 polypeptide fragment (531bp) is soluble, unlike the product of the 2kb fragment. HisTag positive bands were observed between 28 and 188kDa, although no distinct protein band was seen at the expected size (38kDa). Using anti-MCF antiserum (Figure 4.21), again proteins of between 28 and 188kDa were detected. Similar to the findings with Ov8.5 and ORF65, this could suggest a level of non-specific histidine binding in the crude lysate. Therefore purification of the RTS extract was required. Work on the Ov8.5 and ORF65 proteins indicated that purification using the Ni-NTA affinity chromatography was ineffective and therefore the Ov8 exon 1fragment was purified by an NHS-HisTrap column and gel filtration.

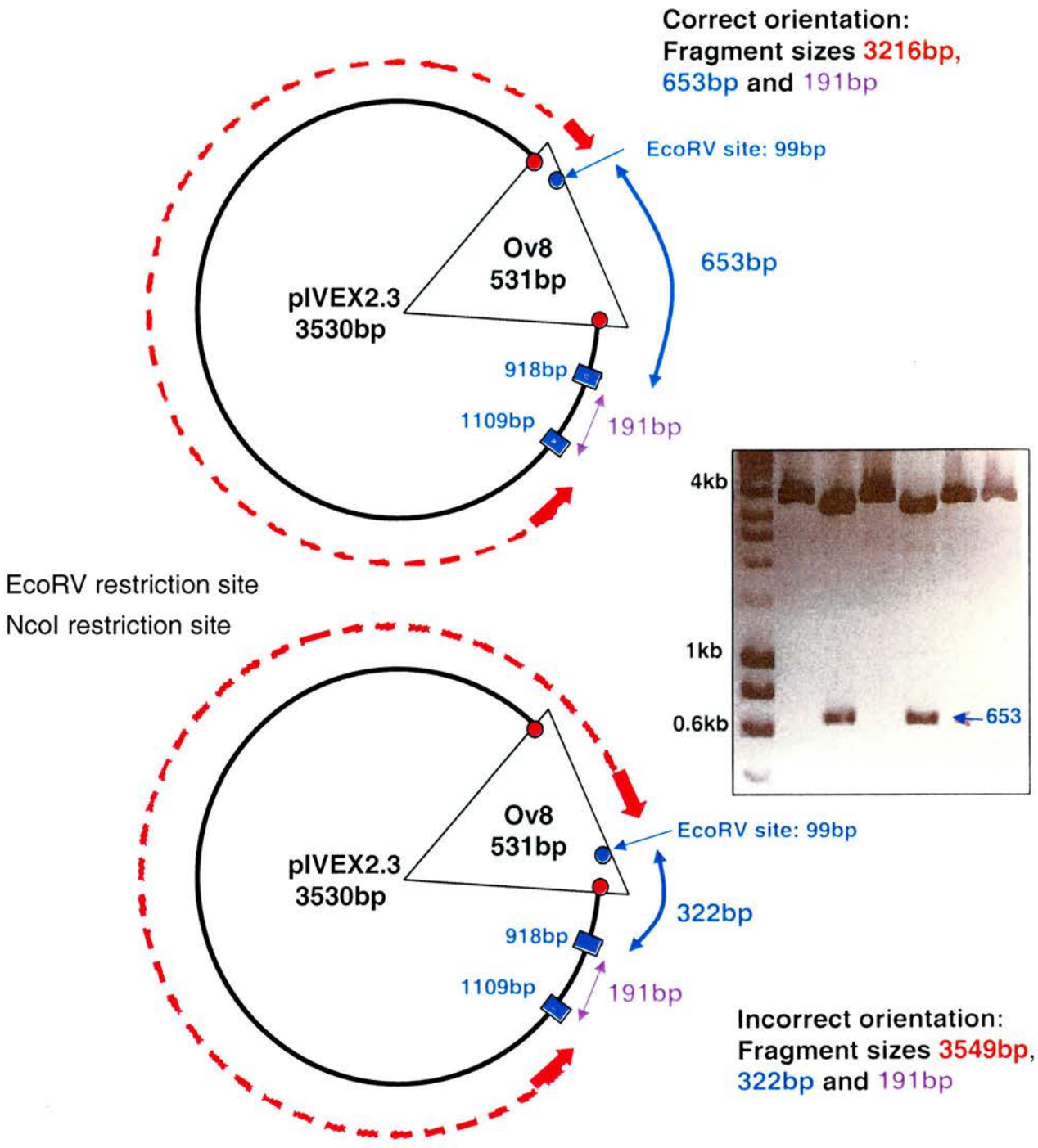


Figure 4.20: Orientation of pIVEX2.3-O8 (0.5 kb).

Presence of insert demonstrated and orientation determined by restriction digest reaction with enzyme EcoRV (▬). Restriction sites in pIVEX2.3 vector at 918bp and 1.1kb (3547bp), and 234bp in the orf65 insert (2.1kb). Result in three bands at 3.1kb, 2kb and 191bp. ● represents NcoI restriction site, Hyperladder I used as DNA marker, Bioline.

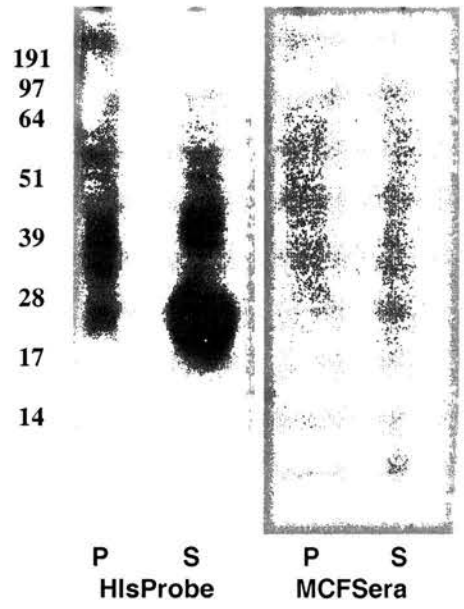


Figure 4.21: Chemiluminescent western blot of pIVEX2.3-Ov8 (Exon 1) RTS extract. Expression of the electrophoresed soluble (s) and insoluble (p) fractions of the pIVEX2.3-Ov8 exon 1 RTS extract, were analysed by western blotting using (a) HisProbe-HRP, (b) MCF-positive antiserum, using a rabbit anti-bovine HRP conjugate. The bound conjugate was detected by chemiluminescence using the Pierce Supersignal West Dura HRP substrate.

4.3.5.1. Affinity purification of pIVEX-Ov8 (0.5kb)

RTS extract of Ov8 exon 1 (100µl) was applied to the HisTrap column under denaturing conditions, using the Atka FPLC system. Twenty-four 1ml eluted fractions were collected. Fractions corresponding to protein peaks on the FPLC system trace were difficult to assign due to the presence of imidazole, so that the seven fractions of the peak were analysed by staining and western blot. The stained gel showed no protein bands in any fraction, although bands were clearly observed in the RTS extract and in the fall-through between 38kDa and 97kDa (Figure 4.22a).

Analysis of the same fractions by western blotting, using HisProbe-HRP antibody, was similar to the stained gel with protein expression observed in both the RTS extract and fall-through supernatant. However, a faint band at approximately 97kDa was observed in fractions 12 and 24 (Figure 4.22b).

4.3.5.2. pIVEX2.3-OV8 gel filtration

Since the initial HisTrap, native and denaturing, purification of Ov8 Exon 1 fusion protein proved ineffective, a further purification technique was performed. Gel filtration was used to fractionate lysates containing the His-tagged fusion protein according to size. Separation was performed on a $Superdex_{200}$ column, using the Akta FPLC system. 0.5ml of pIVEX2.3-Ov8 exon 1 RTS extract was injected onto the column and 1ml fractions were collected and analysed. Thirty fractions were collected and fractions which corresponded to the four protein peaks on the UV trace (Figure 4.23) were analysed by staining and western blotting, using HisProbe-HRP antibody (Figure 4.24) to detect the presence of pIVEX2.3-Ov8 Exon 1 protein in the eluted fractions.

The initial two peaks, fractions 8, 9 and 13, showed faint protein bands ranging from 40 to 97kDa. The third peak, corresponding to fractions 15, 16 and 17, showed three distinct bands at 38, 50 and 64kDa. Fractions corresponding to the third fractionation peak, fractions 20 and 21, showed no protein present. The expected size of the pIVEX-Ov8 gene product is 26kDa which was not shown in any fraction analysed (Figure 4.24a).

A western blot, using HisProbe-HRP antibody, of the same fractions detected one His-Tagged band at approximately 64kDa in size (Figure 4.24b), present in the second peak (fraction 13), which corresponds to a band shown by staining (Figure 4.24a). Although this tagged protein may not be Ov8, it is possible that the Ov8 fragment was migrating aberrantly, or was covalently modified. It is however

surprising that only one band was detected in the fractionated lysate while many bands appeared to be detected in the crude RTS product.

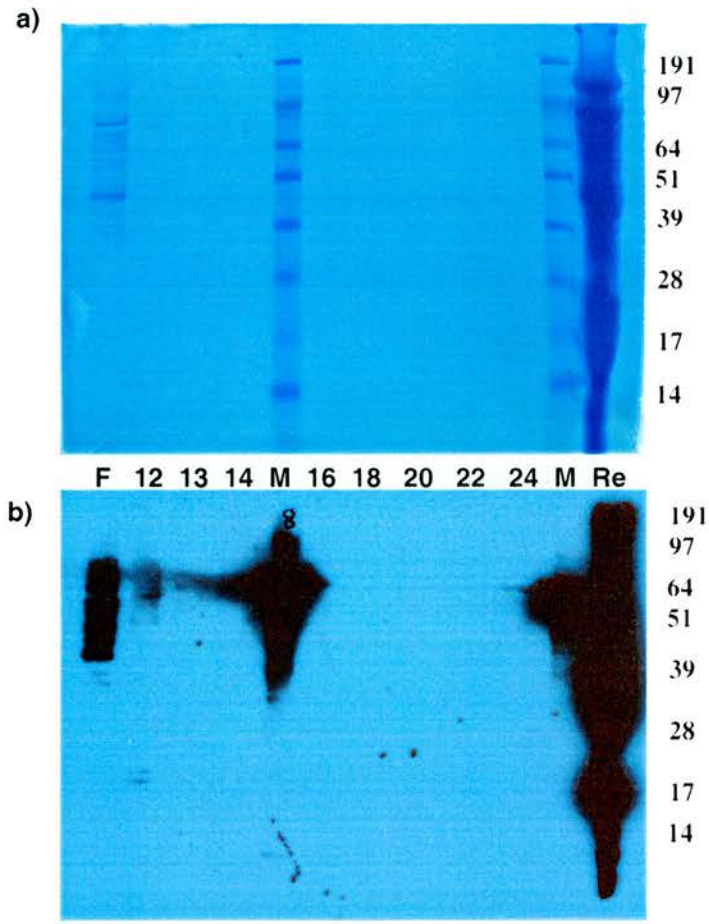


Figure 4.22: HisTrap purification of pIVEX2.4a-ORF65 under native conditions. Fractions (12-24) were electrophoresed alongside the RTS extract (Re) present as a control, and unbound material (F). The electrophoresed fractions were analysed by a) staining with SimplyBlue SafeStain and b) western blotting using HisProbe-HRP antibody. The bound conjugate was detected by chemiluminescence using the Pierce Supersignal West Dura HRP substrate.

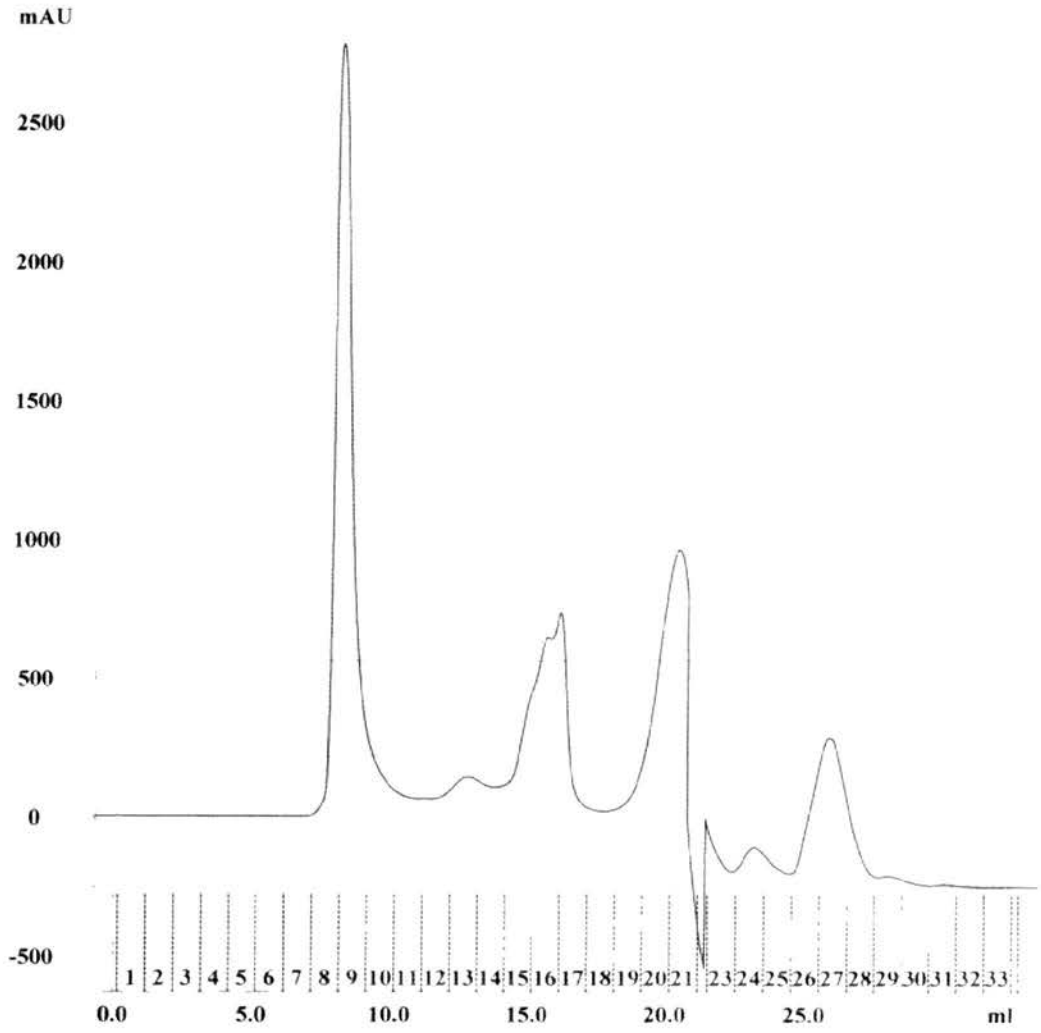


Figure 4.23: pIVEX2.3-Ov8 Exon 1 (0.5kb) chromatogram.

Gel filtration of pIVEX2.3 Ov8 Exon1 0.5kb. Fractions collected in 1ml aliquots from the FPLC system UV trace (280nm).

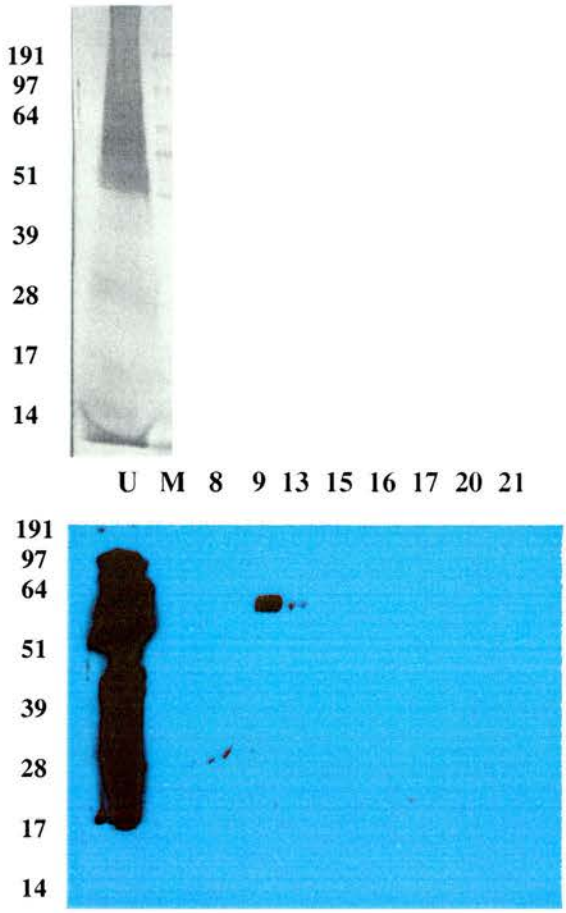


Figure 4.24: pIVEX2.3-Ov8 Exon 1 Gel Filtration.

Fractions (9-13) were electrophoresed alongside the unbound material (U) present as a control. The electrophoresed fractions were analysed by a) staining with SimplyBlue SafeStain and b) western blotting using HisProbe-HRP antibody. The bound conjugate was detected by chemiluminescence using the Pierce Supersignal West Dura HRP substrate.

4.4. Discussion

Initial work on OvHV-2 genes using bacterial expression systems showed that genes such as ORF65 and Ov8 Exon 1 could be expressed. However, due to problems with solubility, purification and isolation of target proteins, an alternative system was sought to allow the antigenicity of the proteins to be evaluated. Three OvHV-2 genes (Ov8, Ov8.5 and ORF65) were analysed using the high-yield cell-free *in vitro* Rapid Translation System (RTS).

The *in vitro* synthesis of proteins in cell-free extracts is an important molecular tool with a variety of applications, including the rapid identification of gene products by proteomics and protein folding studies. The *in vitro* translation systems can have advantages over *in vivo* gene expression when the over-expressed product is toxic to the host cell, such as the OvHV-Ov9 gene in the thioredoxin expression system, described in section 3.3.1. Further advantages include when the product is insoluble (pET22b-Ov8, pTrxFus-Ov2.5, pTrxFus-Ov7) or when the protein undergoes rapid proteolytic degradation (pTrxFus-Ov8). The RTS system showed successful expression of all OvHV-2 genes tested as detected by western blotting of RTS extracts by anti-HisTag antibodies.

The Ov8.5 gene is unique to OvHV-2 with no homology to any other viral or cellular protein (Hart *et al.*, 2007) and appears to be expressed (Thonur *et al.*, 2006). Two tandem repeats are contained within the N-terminal coding region of Ov8.5, at the same position as a number of other gammaherpesviruses such as bovine herpesvirus-4 and Kaposi's sarcoma herpesvirus, where the underlying DNA sequences are involved in the lytic origin of replication (Zimmermann *et al.*, 2001; Hart *et al.*, 2007). Further studies are required to assign a function for Ov8.5.

In previous expression studies using the thioredoxin bacterial expression system, the OvHV-2 Ov8.5 fusion was not expressed. In the RTS system, however, pIVEX2.3-Ov8.5 was detected in the RTS extract. pIVEX2.3-Ov8.5 expressed four bands at 12, 20, 30 and 36kDa, rather than the expected single band at 40kDa, which may be due to protein degradation. It is unlikely that all these bands are Ov8.5 derived, and may suggest non-specific protein interaction. Bands of similar sizes were not shown in any other pIVEX2.3-Ov8.5 western blots. Purification of the Ov8.5 protein, using standard affinity chromatography proved partially successful as the 36kDa fraction was determined by western blot. No band was visible after

Coomassie staining. As only a low level of Ov8.5 protein was expressed, further analysis could not be performed.

The capsid protein ORF65 is likely to be expressed during the lytic cycle of viral replication. This gene is homologous to the HHV-8 viral capsid antigen ORF65 and the EBV gene EFRF3 and is assumed to share related biological functions (Lin *et al.*, 1997). ORF65 was found to be expressed and soluble in the thioredoxin fusion expression system. Purification by standard metal-affinity chromatography was unsuccessful, which may reflect either low expression of the pTrxFus-ORF65 protein or poor affinity for the resin. A further possibility may be poor accessibility of the epitope tag.

Initially the ORF65 gene was cloned into the pIVEX2.3 vector, with a C-terminal HisTag. pIVEX2.3-ORF65 showed the soluble expression of a range of bands, using an anti-HisTag monoclonal antibody and MCF-positive and -negative serum. This indicates a level of histidine binding in the crude lysate as a possible result of *E. coli* proteins. Affinity chromatography was performed under both native and denaturing conditions, but proved unsuccessful in the enrichment and purification of ORF65. A possible explanation for this result may be that the HisTag was not exposed to the column, as a result of protein folding. The ORF65 gene was therefore cloned into a different vector with an N-terminal His_6Tag.

Similar to pIVEX2.3-ORF65, the pIVEX2.4a-ORF65 protein product was found in the soluble fraction using an anti-HisTag monoclonal antibody, however, affinity chromatography proved ineffective in the enrichment and purification of ORF65. The requirement for protein purification from contaminating *E. coli* proteins meant that this ORF65 protein was not investigated further as a potential recombinant diagnostic antigen. Further experiments are required to analyse ORF65 expression and protein structure. However, due to the time constraints, further analyses of the capsid protein were not continued.

In bacterial systems pBAD, pET and the thioredoxin (pTrxFus) fusion expression system vector, the Ov8 exon 1 2.1kb was insoluble. In the RTS vector pIVEX2.3 this 2.1kb protein was detected and shown as insoluble. Extraction of the insoluble protein using different detergent methods showed a level of non-specific bands, detected using both anti-HisTag monoclonal antibody and MCF-positive sera. Therefore as this protein was insoluble, further analysis using MCF-positive and -

negative serum was not performed to demonstrate the antigenicity of the Ov8 exon 1 2.1kb protein detected.

Similar to pTrxFus-Ov8 Exon 1, however, pIVEX2.3-Ov8 Exon 1 0.5kb expressed a soluble protein, at the size expected for the Ov8 fusion protein. As standard affinity chromatography proved ineffective in the purification of all the OvHV-2 candidate genes Ov8.5 and ORF65 including pIVEX2.3-Ov8 Exon 1 (0.5kb), the Ov8 Exon 1 RTS extract was additionally fractionated by gel filtration. Analysis by western blot, using an anti-HisTag monoclonal antibody showed several tagged bands although none at the size expected for the pIVEX-Ov8 (exon 1) fusion product.

Purification of the candidate proteins is required due to *E. coli* background interference before analysis of their suitability in a diagnostic assay. The use here of the Roche rapid translation system showed that ORF65 and OV8 exon 1 could be expressed as soluble proteins, similar to their expression in one or more bacterial systems. Unfortunately, neither of the polypeptides could be purified by affinity chromatography suggesting the His-tag epitope is not an ideal purification tag for this work. The RTS approach did not improve the solubility of OV8 (2kb), although it was expressed at high levels. This protein, however, could not be solubilised by any approach and could not be studied further. The RTS system, however, proved effective in the expression of OV8.5, which was previously not expressed in the bacterial thioredoxin expression system.

The inability to express or purify selected candidate genes by the RTS approach suggests that bacterial expression may not be the best system for overexpression of these viral genes, despite its ease of use and performance with other genes. Expression by eukaryotic systems, such as yeast, insect or mammalian cells, may prove to be better methods to express viral genes for analysis of their antigenic behaviour. Of particular interest to analyse further is the capsid protein ORF65 which has been shown to be antigenic in the *In Vitro* system, although further studies must be performed to confirm this finding.

In the absence of good recombinant antigen expression, alternative methods for identifying potential diagnostic antigens were followed, including the use of crude antigen preparations from cultured AlHV-1 and OvHV-2 infected cells, analysed by both ELISA methods and western blotting.

Chapter Five

Establishment of the WC11-ELISA

5.1. Introduction

MCF typically emerges sporadically, with a small number of affected animals, although epidemics may occur (Brenner *et al.*, 2002). Outbreaks where several animals in a herd were infected have been reported in Spain (Yus *et al.*, 1999), Turkey (Dabak and Bulut, 2003), Saudi Arabia (Abu Elzein *et al.*, 2003) and Great Britain (Hamilton, 1990; Collery and Foley, 1996; Otter, Pow and Reid, 2002). Generally, the disease is of low prevalence, although herd outbreaks have shown as many as 50% of animals can be affected. The most common characteristics of the disease include a high temperature, nasal and ocular secretions, followed in the majority of cases by death. As MCF has a range of clinical signs and is similar to a variety of other diseases, initial diagnosis can prove difficult (Holliman, 2005).

MCF infections in cattle can result in clinical signs within weeks although in some cases they may take months to develop (O'Toole *et al.*, 1995 and 1997; Brenner *et al.*, 2002). Once an animal presents with clinical signs, mortality approaches 100%. There is, however, evidence for animals with chronic MCF recovering (Milne and Reid, 1990; Otter *et al.*, 2002). In addition to these findings, further studies have shown animals from an MCF outbreak that died without showing any characteristic signs of MCF (Brenner *et al.*, 2002). This suggests that cattle may become infected without developing clinical MCF (Powers *et al.*, 2005).

The identification of MCF affected animals is currently based on the detection of antibodies to AlHV-1 antigens using a variety of assays including virus neutralisation and indirect-immunofluorescence tests (Plowright *et al.*, 1963; Office International des Epizooties, 2000), although histopathological examination remains the most recognised diagnostic procedure to date (Muller-Doblies *et al.*, 1998). PCR assays are also available for the detection of MCFV (Baxter *et al.,* 1993; Hussy *et al.*, 2001; Flach *et al.,* 2002; Li *et al.,* 2005).

Virus neutralisation (VN) and immunofluorescence tests (IFAT) have been employed to determine the MCF status of animals and for the confirmation of MCF in susceptible species. Of these tests, the virus neutralisation test is the most virus-specific (Wan *et al.*, 1988) as indirect immunofluorescence tests may detect antibodies to other bovine herpesviruses e.g. bovine herpesvirus-4 (BoHV-4). By immunofluorescence, sera are only considered positive when foci show characteristic intra-nuclear distribution of antigen with little or no cytoplasmic staining being detected in the AIHV-1-infected cells (Wan *et al.*, 1988; Li *et al.*, 1994). No reaction

should be observed in BoHV-4 infected cells, therefore, a negative control for this test should include cell monolayers infected with BoHV-4.

As a basis for an MCF-specific assay, a competitive-inhibition ELISA (CI-ELISA) was developed (Li *et al.*, 1994) based upon a monoclonal antibody (MAb 15-A) which binds an epitope, on a complex of glycoproteins, conserved among all MCFV isolates. The Minnesota MCF isolate virus used as the coating antigen for the CI-ELISA antigen, is indistinguishable from the WC11 strain of AlHV-1 (*Office International des Epizooties*, 2003). Although specificity proved high (91-100% relative to IFAT), analysis of serum samples using the CI-ELISA proved less sensitive (56-87%) than diagnosis based either on histopathology or PCR assay results (Muller-Doblies *et al.*, 1998). The CI-ELISA sensitivity was later improved in a reformatted ELISA, conjugating the monoclonal antibody directly with horseradish peroxidase to offer a four-fold increase in analytical sensitivity (Li *et al.*, 2001).

Although the CI-ELISA has the advantage of being faster and more efficient than IFAT (*Office International des Epizooties*, 2003), disadvantages of the test include the potential for false negative results, where antibodies specific to the conserved epitope have not developed in an infected animal. Additionally, the cost of the CI-ELISA may explain why the assay is not widely used, especially in developing countries ($250 per plate excluding shipping costs). The provision of a simple MCF-antibody specific ELISA would therefore prove beneficial in the routine surveillance of clinical cases and in the study of MCF in sheep and cattle which remain asymptomatic.

The principle aim of this study was to test whether an ELISA for MCFV-specific antibodies, based on a crude infected-cell lysate, could provide a diagnostic test that may be universally available. Initial stages required in the development of a new, diagnostic assay such as an ELISA consist of feasibility studies for protocols and reagents used. Standardisation of the test and its performance should also be examined prior to tests being routinely used (Office International des Epizooties, 2003).

The MCFV strains (WC11 (Plowright *et al.*, 1963) and C500 (Plowright *et al.*, 1975), derived from wildebeest, and the OvHV-2 BJ1035 cell line derived from sheep (Shock *et al.*, 1998) were analysed as possible assay antigen reagents (described in section 2.7.3). AlHV-1 and OvHV-2 are antigenically related so that cross-reactivity in MCF serology occurs (Rossiter, 1981: Reid *et al.*, 1989). Positive

and negative control serum and conjugated antibody solutions were analysed, and optimal concentrations of each were determined in the absence of diagnostic samples with a view to designing an MCFV ELISA (section 5.2.1.1).

This chapter describes the development of an AlHV-1 ELISA for MCFV-specific antibodies based on a crude infected-cell lysate, for the detection of anti-MCFV antibodies and compares the results obtained with established diagnostic assays. To complete the establishment and preliminary validation of this assay, serum samples submitted for MCF testing were collected and analysed in a parallel study to allow a comparison of IFAT, the commercially available MCF ELISA (CI-ELISA) and the virus-based WC11-ELISA. The WC11-ELISA was further used to examine the prevalence of antibodies to OvHV-2 in cattle with clinical MCF and examine the potential of cross-reactivity to other bovine herpesviruses.

5.2. Materials and Methods

5.2.1. ELISA antigen preparation

Due to the lack of a productive cell culture system for OvHV-2, AlHV-1 infected cell culture was analysed. A second reason for the WC11 isolate being analysed was due to the same strain being used in the diagnostic test, indirect immunofluorescence assay (IFAT) and the availability of a suitable control antigen.

Four 150cm^2 flasks of BT cells were washed twice with Hanks balanced salts solution (HBSS). Two flasks were inoculated with 4mls (4×10^5 $TCID_{50}$/ml) of cell culture-grown virus WC11 supernatant, while the remaining two flasks were inoculated with 4mls of HBSS and used both as a negative control and to prepare the negative coating antigen. All flasks were incubated for one hour at 37°C, with occasional rocking to ensure even distribution of inoculum. Fifty millilitres of IMDM (Gibco Invitrogen cell culture, Paisley, UK) medium supplemented with 10% (v/v) FBS, 1% (w/v) glutamine and 1% (w/v) penicillin with streptomycin (100µg/ml) were added to each flask and the flasks were incubated at 37°C until 90-100% CPE was observed in the infected flasks. CPE was seen after 4-5 days.

The supernatant, containing infected and detached cells, was decanted into 50ml centrifuge tubes. Fifty millilitres of 1% PBS (v/v) was added to each flask to wash remaining cells, and then decanted and centrifuged at 2000 x g for 10 minutes at 4°C. The infected cell pellet was re-suspended and pooled into 100mls of PBS and re-centrifuged. The supernatant from each tube was discarded and the cell pellets re-suspended in 1.5ml of 1% PBS (v/v) containing 1% Nonylphenylpolyethylene glycol (Nonidet-P40; NP40) detergent (Merck Bioscience, Nottingham. UK), and placed back into the original cell culture flask. The flasks were incubated for two hours at 4°C, tilting every 15 minutes, to remove and lyse attached cells. Soluble lysate was removed from each flask, pooled and clarified at 11,600 x g for 5 minutes at room temperature (22 ± 5°C). Supernatant was removed and the pellet discarded. The supernatant was stored at -70°C until required. As the cells were not detached in the control flasks, supernatant was removed and PBS containing 1% NP40 placed to the flasks to lyse attached cells. ELISA antigen was also prepared using the AlHV-1 C500 virus isolate by the same method. Virus inoculum was kindly supplied by the Virus Surveillance Unit, Moredun Research Institute.

The BJ1035 LGL line from an OvHV-2 infected animal (Shock *et al.*, 1998) was maintained in IMDM supplemented with antibiotics (as described in section 2.3.1.) and 350 IU/ml of IL-2 (Proleukin, Chiron Therapeutics, Emeryville, CA).

Medium from two $75cm^2$ flasks containing the cell line was decanted, when confluent, into 50ml centrifuge tubes. Cells were non-adherent. Ten millilitres of PBS was added to each flask to wash the remaining cells, and decanted into centrifuge tubes. Cells were harvested and centrifuged at 2000 x g for 10 minutes at 4°C. The cell pellets and cells remaining in the flasks were then treated as stated for the AlHV-1 ELISA antigen.

As there was no simple negative control cell line available for the OvHV-2 BJ1035 cell line, ConA blasts were produced as an appropriate uninfected control. Blood was collected in preservative-free heparin (200µl/10ml) from a MCF-negative calf. Under sterile conditions, the blood was centrifuged at 1800 x g for 10 minutes (22± 5°C). The white cell layer was removed, and was resuspended in 5mls of HBSS supplemented with 1% FBS and penicillin and streptomycin. The cells were then layered onto 5mls of lymphoprep (Fresenius, Oslo, Norway) in a 15ml conical tube and centrifuged at 2000 x g for 45 minutes (22± 5°C) without active braking. The lymphocyte layer was removed at the interface between the lymphoprep and the medium, resuspended in 20mls of wash medium and centrifuged at 1800 x g for 10 minutes. The cell pellet was resuspended in 5mls of medium and a viable cell count was performed (optimum concentration of 1×10^6 cells/ml required). The cells were pelleted and resuspended in IMDM medium supplemented with antibiotics. Cells were then inoculated onto 6-well plates, 2×10^6 cells/well with 5 µg/ml of ConA and incubated at 37°C in 5% CO_2 until cell aggregation was observed. Cells were harvested and centrifuged at 2000 x g for 10 minutes at 4°C (described in section 2.6.4.1.2). The soluble lysate of these cells was stored at -70°C until required.

5.2.2. ELISA Optimisation

Titrations to determine optimum reagent concentrations were carried out under standard test conditions as described by Hebert *et al.*, (1985). Optimum reagent concentrations were established by varying the concentrations of antigen and HRP-conjugated antibody and primary serum antibody. Firstly, the antigen and conjugate was optimised using a 1/100 serum dilution. On establishing optimised conditions, the test serum dilution was optimised.

The concentration of virus-infected antigen employed was determined by BCA protein assay as 0.5mg/ml. Aliquots of infected AlHV-1 antigen (100µl) were coated onto columns 1, 3, 5, 7, 9 and 11, while aliquots of the BT cell line, used to

propagate the virus and as a control antigen, were coated onto columns 2, 4, 6, 8, 10 and 12 of a M129B microELISA plate (Greiner Bio-one, Frickenhausen, Germany). To determine the optimal antigen concentration, the AlHV-1 antigen was diluted in carbonate coating buffer (0.2M Na_2CO_3, 0.2MNaHCO$_3$, pH9.6). Dilutions ranged from 1/50 to 1/850. The plate was incubated overnight at 4°C. The negative control was included to differentiate the specific anti-viral antibody from antibodies directed against antigenic determinants of the cell line used, and therefore aims to reduce the number of false positives and establish background reactivity for each antiserum.

Between each stage the plates were washed four times in ELISA wash buffer (PBS plus 0.05% Tween-20, [PBST]) using an automated ELISA plate washer (Ultrawash plus, Dynatech laboratories).

All subsequent dilutions were made in PBST containing 10% (v/v) horse serum (PBSTH). One hundred microlitre volumes were used. Control MCF positive serum, at a 1/100 dilution, was added to all wells. The plate was incubated for 1 hour at 37°C. After washing, conjugate (Donkey anti-sheep or Rabbit anti-bovine IgG horseradish peroxidase [Sigma, Poole,UK]) was added at dilutions ranging from 1/500 to 1/5000, to duplicate infected and control wells. The plate was incubated for a further one hour at 37°C, washed, and 100µl tetramethylbenzidine (TMB) peroxidase substrate 1-component added (KPL Insight Biotechnology Limited, Middlesex, UK). The reaction proceeded at room temperature (22±5°C) for 5 minutes before being stopped by the addition of 100µl 0.18M H_2SO_4. The optical densities were read using a MRX plate reader (DYNEX Technologies, Virginia, USA) with a 450nm filter. Results were calculated as an average antigen-specific optical density, i.e. average OD of infected antigen paired samples minus average OD of control antigen paired samples.

The tests were deemed valid if there was no significant difference between the positive and negative controls, performed as standard on each plate tested. Additionally no significant difference was noted between the first and last plate read, and the time between reading plates was minimal.

Two horseradish-peroxidase (HRP) conjugates were tested to determine the optimum conjugate conditions for an AlHV-1 MCF ELISA. Donkey anti-sheep IgG monoclonal antibody (The Binding Site Limited, Birmingham, UK) was tested as it is used routinely in the indirect immunofluorescence assay. In addition, Rabbit anti-bovine IgG polyclonal antibody (Sigma) was tested under the same conditions.

5.2.3. Test Serum

Three positive serum controls, from cattle experimentally immunised with attenuated AlHV-1, were tested (K265, K525 and K856). These samples were MCF seropositive by IFAT. A positive control serum for OvHV-2 (05-687) was collected from a cow presenting clinical signs of MCF, positively diagnosed by IFAT. Serum collected from a clinically healthy cow (700738) was used as a negative control in the ELISA. All test serum was kindly obtained from Iris Campbell (MCF group vaccination study) and Janice Gilray, Virus Surveillance Unit, MRI.

To address potential cross-reactivity with other herpesviruses in the WC11-ELISA, reactivity against bovine herpesvirus-4 (BoHV-4), infectious bovine rhinotracheitis virus (IBR/BHV-1) and bovine herpesvirus-2 (BHV-2/bovine mammilitis) sera was tested. BHV-1, BHV-2 and BoHV-4 BHV serum samples were kindly obtained from Janice Gilray, Virus Surveillance Unit, MRI. These samples were from field cases submitted for routine diagnostic testing by the surveillance unit.

5.2.4. Positive Reaction Threshold

To establish the range of negative reaction values and a threshold positive reaction in the WC11-ELISA, negative serum control samples from 197 clinically healthy cattle, originating from a farm with no history of MCF and no direct contact with sheep, were kindly provided by George Caldow of Scottish Agricultural Colleges Veterinary Services. Each sample was tested in duplicate.

5.2.5. Analysis of Field Sera

Using optimum reaction conditions for antigen, control serum and conjugated antibody, field sera were analysed. Samples were taken from field sera submitted in 2004 for the detection of antibodies against MCFV by IFAT. These included cattle with clear signs of MCF and others with clinical signs only indicative of enteric disease. Sera from cattle with clinical symptoms indicative of potential MCF were submitted to the Virus Surveillance Unit at the Moredun Research Institute, for routine testing by IFAT. Serum samples which tested IFAT negative were also included for analysis. Clinical evaluation and histopathological testing were carried out to provide a final diagnosis in some cases. All sera were stored at -20°C until required. Each sample was tested in duplicate and any discrepant results were re-analysed. As the IFAT values were already known for the sera samples tested, all

sera were tested blind. This was achieved by coding all the samples randomly. Some serum samples tested IFAT positive.

5.3 Results

5.3.1. Development of an Enzyme-linked Immunosorbent Assay

5.3.1.1. Determination of optimum ELISA reaction conditions: AlHV-1 MCF antigen, Horseradish peroxidase conjugate and test serum.

In order to determine the optimum conditions for an MCFV ELISA, the concentrations of all reagents were titrated against each other.

As the Minnesota MCF isolate virus, used as the coating antigen for the CI-ELISA antigen, is indistinguishable from the WC11 strain of AlHV-1, routinely used by IFAT (*Office International des Epizooties*, 2005), the WC11 antigen was initially tested. The antigen was tested at dilution from 1/50 to 1/300 (Figure 5.1). The control antigen used was mock-infected BT cells. No negative control serum was tested at this initial optimisation. As this test was to determine the conjugate and antigen dilutions to use, no test serum was analysed. Dilutions were tested in duplicate for both positive and negative antigen, whereby the mean of negative antigen was subtracted from the mean of the positive antigen. These results showed no end-point of activity; therefore larger ranges of dilutions were tested to identify the optimum antigen dilution. The positive control serum, K856, was used at an initial 1/100 dilution. All titrations were performed in duplicate.

Two HRP-conjugated antibodies were tested, HRP-labelled Donkey anti-sheep monoclonal antibody and Rabbit anti-bovine IgG polyclonal antibody. The conjugated antibodies were tested at dilutions ranging between 1/2000 and 1/5000 to gain an estimated HRP-conjugate dilution (Figure 5.1). The absorbance values obtained for the Donkey anti-sheep HRP conjugate were relatively low, between 0.2 and 0.8 absorbance units (AU) (Figure 5.1a.). The Rabbit anti-bovine HRP-conjugate showed higher activity, between 0.4 and 1.2 AU (Figure 5.1b.). Diluted at 1/2000, the rabbit anti-bovine IgG conjugated antibody (polyclonal) showed the highest activity (1.2 AU) and was used in further optimisation tests.

The highest AU_{450} values was found at a 1/2000 dilution, therefore in order to determine whether a lower conjugate dilution would be optimal, a dilution range of 1/500 to 1/2000 was analysed. Antigen concentrations were also extended as no reaction end-point was initially reached (1/50 to 1/850). Activity increased rapidly between antigen dilutions of 1/50 and 1/200 (Figure 5.2.) where it peaked at an absorbance of 0.6 AU, and remained relatively constant at approximately 0.5-0.6AU from 1/200 to a dilution of 1/350, before decreasing rapidly to an absorbance of

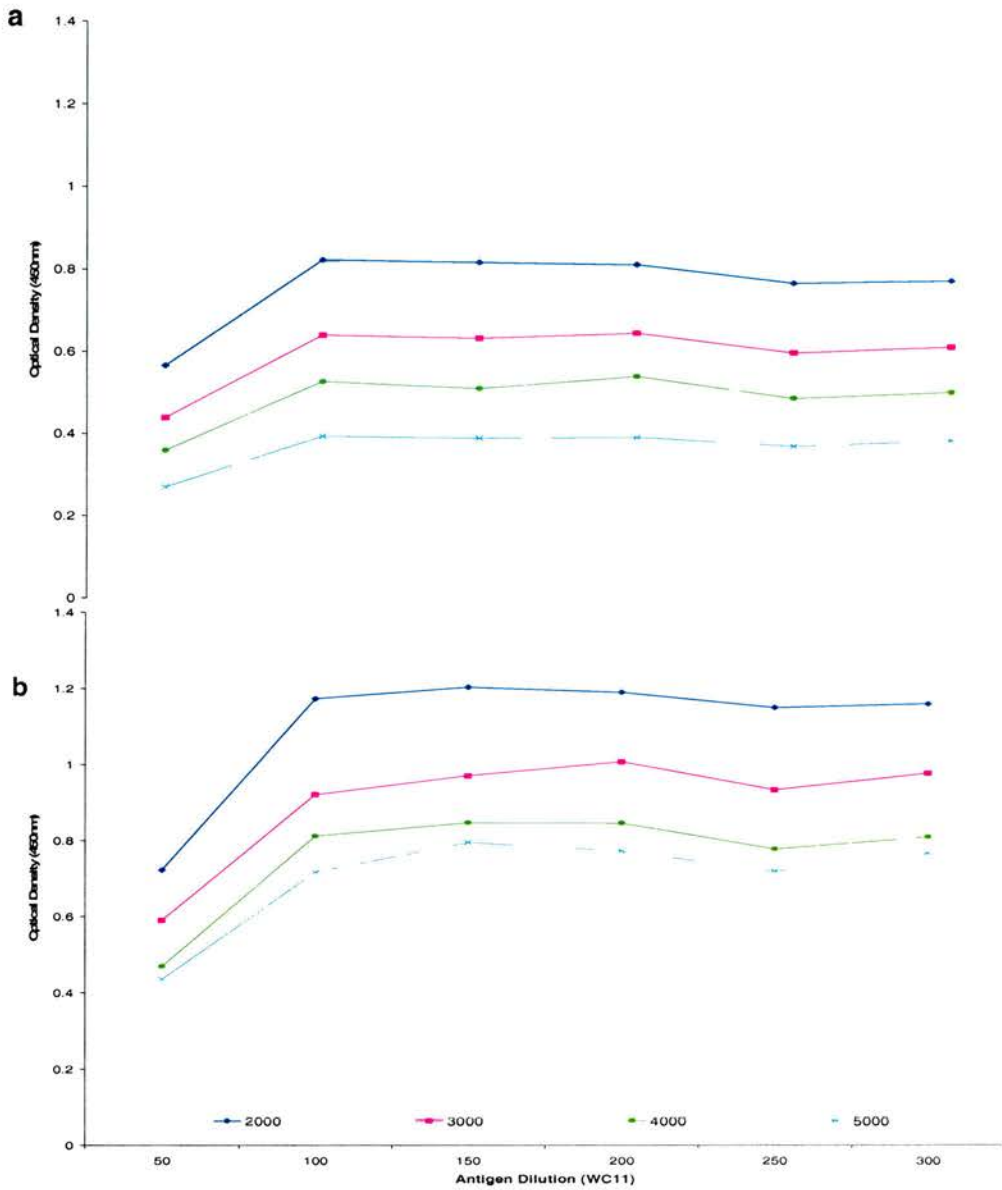


Figure 5.1: Optimisation of MCF direct ELISA. The MCF virus strain WC11and BT cell control were tested at varying dilutions (1/50-1/300) at a range of conjugate concentrations (1/2000 and 1/5000 shown by legend) to determine the optimal test conditions. Control serum (K856) was kept at a constant dilution of 1/100 to acquire the optimal antigen and conjugate reaction conditions. Two HRP conjugates were analysed (a) Donkey anti-ovine IgG and (b) Rabbit anti-bovine IgG conjugate due to being employed in established MCF diagnostic tests. The y axis represents the average OD for the positive antigen minus the average OD for the negative antigen, while the x axis represents antigen dilution.

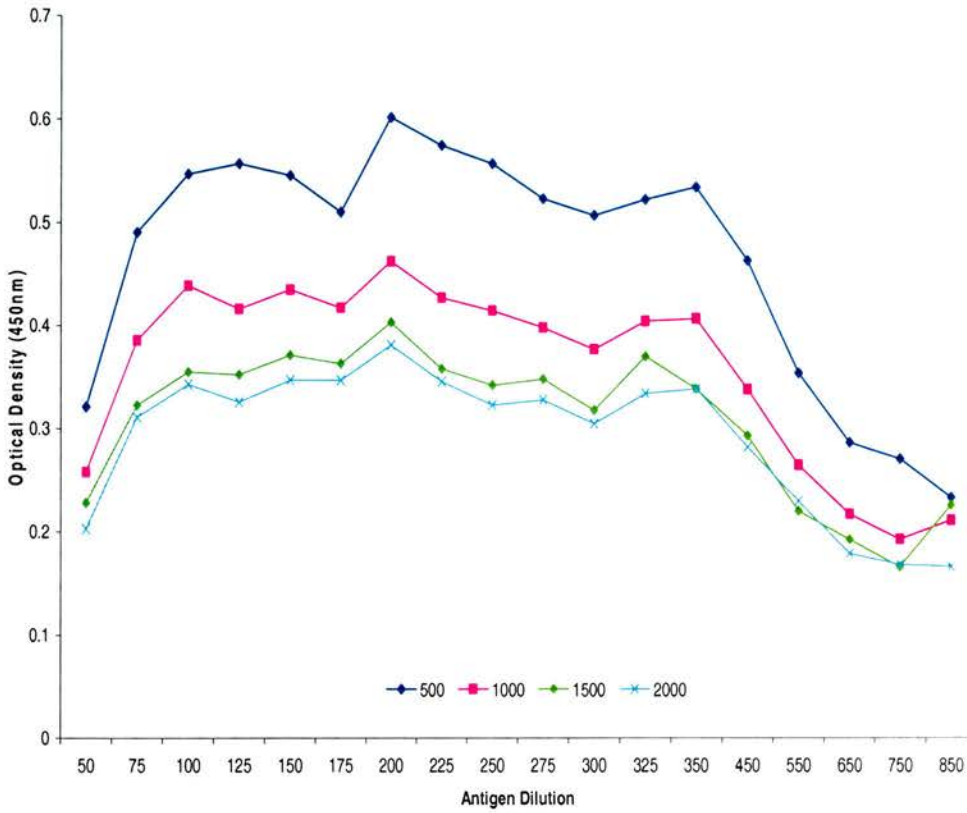


Figure 5.2: Optimisation of MCF direct ELISA conjugate concentration. AlHV-1 virus strain WC11 was tested at varying dilutions (1/50-1/850) at a range of Rabbit anti-bovine conjugate concentrations (1/500 and 1/2000 shown by legend) to acquire the optimal antigen and conjugate reaction conditions. The y axis represents the average OD for the positive antigen minus the average OD for the negative antigen, while the x axis represents antigen dilution.

0.2AU. Discrepancies were noted in the OD values obtained at antigen dilutions 1/200 and 1/350, this can be explained by the use of a new ELISA plate, therefore there may be a difference in absorbance due to the hydration of the individual plate wells, particularly in the outer wells.

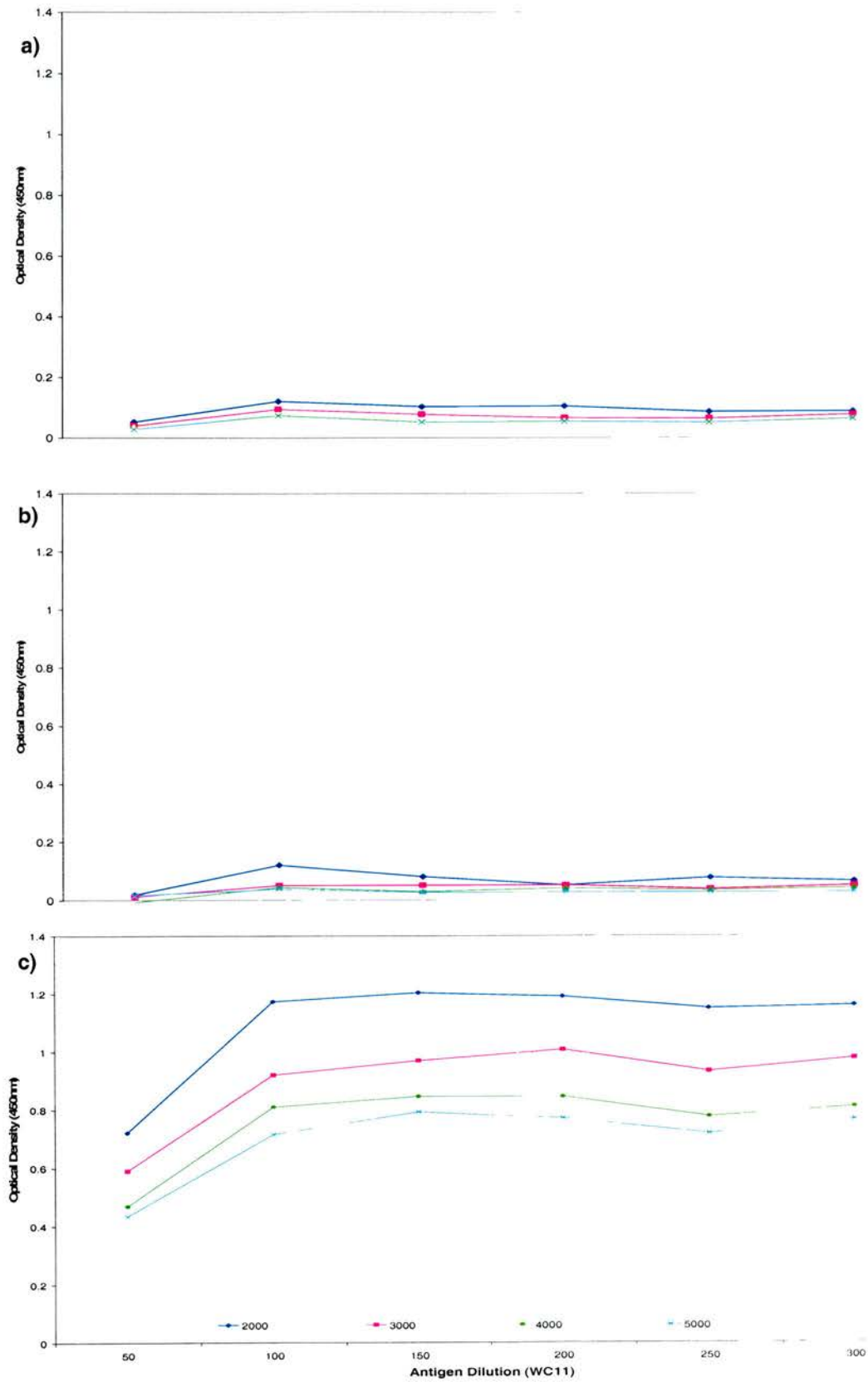
The optimal WC11 antigen concentration selected was 1/200. All dilutions of conjugate showed similar profiles across the range of antigen dilutions, with a 0.1 absorbance reduction between the 1/500 and 1/1000 dilutions; and a further 0.1 absorbance reduction between 1/1500 and 1/2000, while 1/1500 and 1/2000 dilutions showed similar results. The selected reaction conditions were therefore at a conjugated antibody (HRP-rabbit anti-bovine IgG) dilution of 1/500. Discrepant results

Peak absorbance values in optimisation test two were approximately half that of the previous test (0.6 AU); this may be explained as a result of leaving the antigen coated plates an extra 24 hours (+4°C) before washing and adding serum samples. Although the greatest activity was at a 1/500 dilution, a more economical conjugate dilution of up to 1/2000 could be employed without altering the outcome.

Using the optimised conditions, three MCF positive serum samples, from experimentally infected animals, were tested. To establish positive control values, a series of antigen dilutions were tested, ranging from 1/50 to 1/300 with conjugate (HRP-rabbit anti-bovine IgG) dilutions ranging from 1/2000-1/5000 using the positive control sera at a 1/100 dilution (Figure 5.3.).

Sera samples K265 and K525 showed an increase in ELISA reaction between antigen dilutions of 1/50 and 1/100 with an absorbance of 0.12 AU. Between 1/100 and 1/200 antigen dilutions, the activity reduced before remaining constant at approximately 0.1AU at a dilution of 1/250 (Figure 5.3a.). The optical density values of these sera samples were low due to non-specific background interference. Serum K856 increased in activity between antigen dilutions of 1/50 and 1/100 (Figure 5.3c.) and peaked at an absorbance of 1.2 AU. At 1/100 activity remained relatively constant at approximately 1.2 AU up to a dilution of 1/300. It was found that the K856 serum showed the greatest activity of the control sera using both conjugates, and was subsequently employed in the optimisation of the basic ELISA reagents. Although the K856 results showed no end-point, an antigen dilution of 1/200 and a conjugate dilution of 1/1000 were employed in subsequent sera tests.

Figure 5.3: ELISA analysis of MCF-positive sera. Positive bovine serum samples (a) K265 (b) K525 and (c) K856, tested at a range of AlHV-1 virus (strain WC11) antigen dilutions (1/50-1/300) at a range of Rabbit anti-bovine conjugate concentrations (1/500 and 1/2000 shown by legend) to acquire the optimal reaction conditions.


a)
b)
c)
Optical Density (450nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2000
3000
4000
5000
50
100
150
200
250
300
Antigen Dilution (WC11)

The positive control serum K856 and negative serum sample from a clinically healthy cow were used for testing with the optimal reaction conditions determined. Both samples had been previously tested by IFAT, confirming the positive/negative status of the samples utilised. In order to determine the optimal concentration of serum required for the test, a series of dilutions were performed (ranging from 1/25 to 1/600) for both serum samples. The data obtained for the positive serum showed a gradual decline in absorbance values, decreasing from an absorbance value of 1.5 (serum dilution 1/25) to 0.6 (dilution 1/600). No reactivity was observed for the negative serum. The optimal reaction conditions were therefore at a serum dilution of 1/25 (Figure 5.4.). Although the greatest reaction was under these conditions, a more economical dilution of 1/100 was employed.

5.3.1.2. Antigen Optimisation

5.3.1.2.1. AlHV-1 C500 strain

To assess other potential ELISA antigens which may be used in a MCF specific diagnostic test, other MCF virus isolates were analysed. The C500 high-passage strain of AlHV-1 was therefore compared against WC11. A range of antigen dilutions (1/50-1/250) and conjugate dilutions (1/500-1/2000) with a serum dilution of 1/100 were analysed. The results showed the same optimal conditions for both AlHV-1 viral strains. IFAT results were used as a gold standard in this case. The C500-ELISA gave a sensitivity of 67% and a specificity of 100% relative to IFAT results, while the WC11-ELISA showed 100% agreement with the IFAT results (Table 5.1). Since C500 was no better as an ELISA antigen than WC11, studies were continued with the WC11 ELISA.

5.3.1.2.2. Sucrose gradient purified virus, strain WC11.

Sucrose-gradient purified AlHV-1 virus ($4x10^5$ $TCID_{50}$/ml), described in section 2.2.4, was additionally analysed as a potential ELISA antigen. A range of antigen dilutions (1/100-1/600) and conjugated antibody dilutions (1/500-1/2000) were analysed, with a serum dilution of 1/100. Results showed no end-point for the reaction (Figure 5.5.). A comparison of purified and crude WC11-infected lysate, under optimal antigen and conjugated antibody dilutions, showed a difference of 0.9 AU between the two methods.

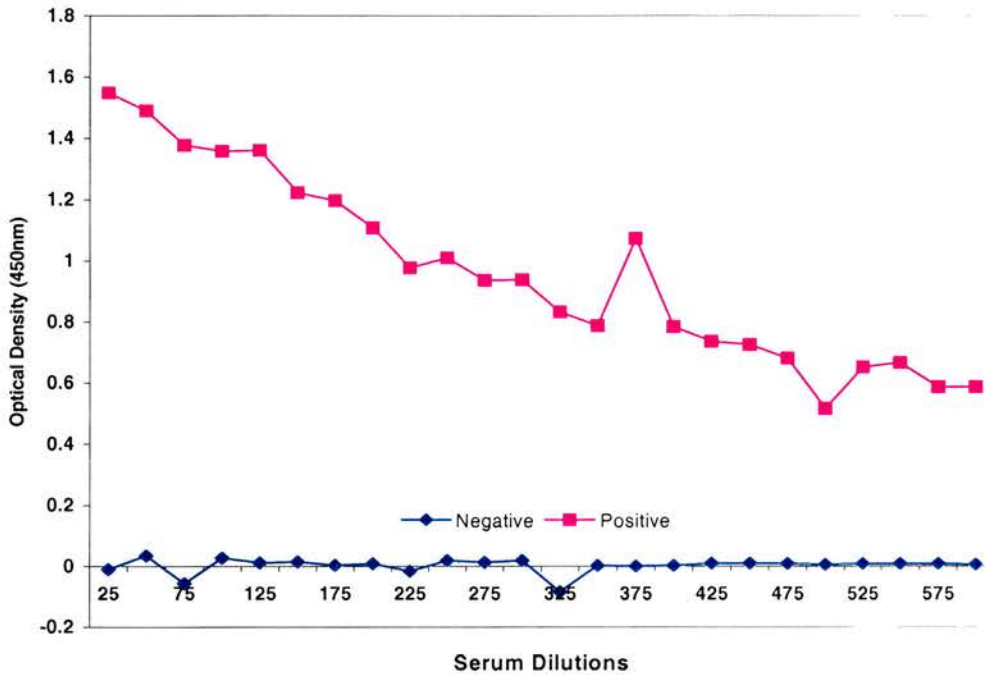


Figure 5.4: Comparison of MCF-positive and negative serum samples.

Dilutions of positive (K856) and Negative (700738) bovine serum samples tested using determined optimal reaction conditions (antigen dilution of 1/200 & conjugate dilution of 1/1000).

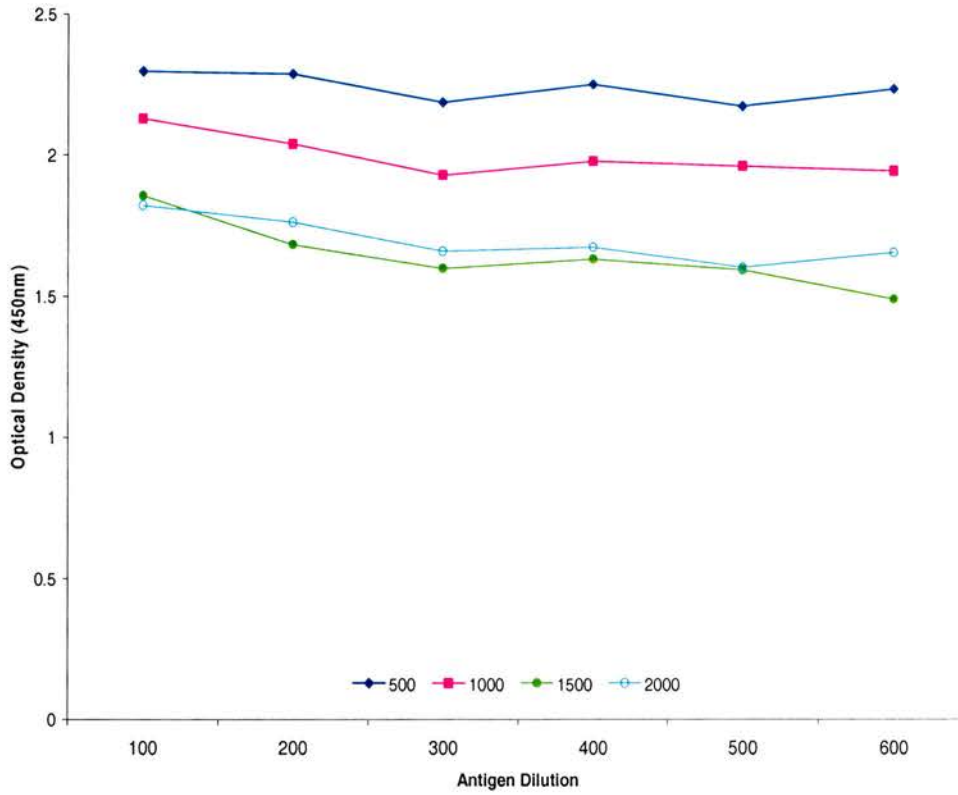


Figure 5.5: Sucrose-gradient purified AlHV-1 virus (strain WC11).

Virus tested at varying dilutions (1/100-1/600) at a range of conjugate concentrations (1/500 and 1/2000 shown by legend) to acquire the optimal antigen and conjugate reaction conditions. A serum dilution of 1/100 was employed.

A peak in activity of 2.1 AU at a 1/100 antigen dilution was shown for the purified virus. Activity showed a gradual decline in absorbance values to 1.9 AU at a 1/200 antigen dilution and remained relatively constant up to a dilution of 1/600. Therefore compared to the crude lysate optimal antigen dilution of 1/200 and conjugate dilution of 1/1000, a more economical antigen dilution of up to 1/600 with a conjugate dilution of 1/2000 could be employed for purified virus. This could result in a loss in sensitivity with a reduction of 0.2 AU in absorbance. This suggests purified virus could be employed at a dilution greater than that of the crude lysate. However, due to the time-consuming protocol required for sucrose-gradient purified virus, this was not seen as a viable source of antigen.

Sample	C500		WC11		IFAT
	Optical Density	Result	Optical Density	Result	Result
1	-0.059	Negative	0.014	Negative	Negative
2	0.19	Positive	0.397	Positive	Positive
3	0.002	Negative	0.259	Positive	Positive
4	-0.005	Negative	-0.028	Negative	Negative
5	-0.05	Negative	-0.065	Negative	Negative
6	-0.006	Negative	-0.062	Negative	Negative
7	-0.053	Negative	0	Negative	Negative
8	0.215	Positive	0.528	Positive	Positive
Positive Control	**0.854**	**Positive**	**0.945**	**Positive**	**Positive**
Negative Control	**-0.049**	**Negative**	**-0.003**	**Negative**	**Negative**

Table 5.1: Analysis of selected MCF bovine field serum samples (n = 8), by the C500-ELISA, WC11-ELISA and IFAT (87.5% agreement).

5.3.1.2.3. Ovine herpesvirus-2

ELISA antigen prepared from OvHV-2 lysate from an infected cell-line (BJ1035) was tested using an OvHV-2 positive (05-687) and negative (700738) control serum. A range of antigen dilutions (1/25-1/600) and conjugated antibody dilutions (1/500-1/2000) with a serum dilution of 1/100 were analysed (Figure 5.6). The OvHV-2 lysate-based ELISA gave optical density values that were greater for the control antigen than the infected cell lysate and was, therefore, not seen as a viable source of antigen (Figure 5.6).

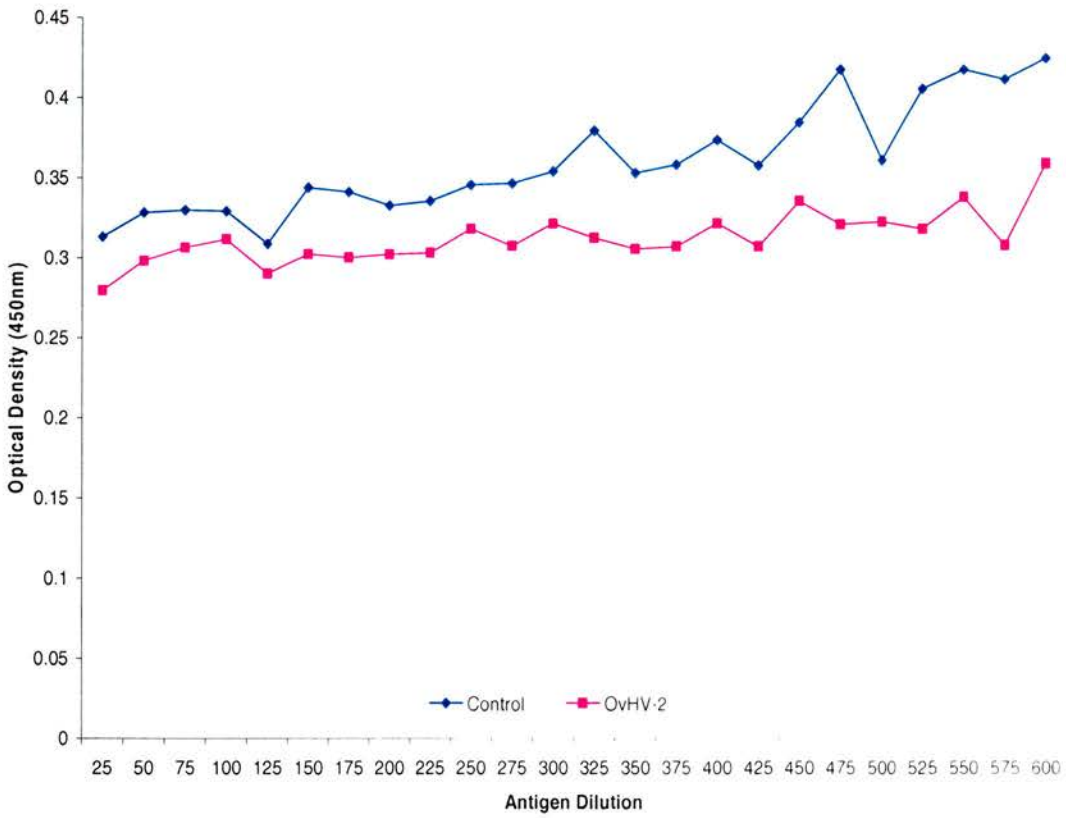


Figure 5.6: Optimisation of OvHV-2 ELISA antigen

OvHV-2 virus tested at varying dilutions (1/25-1/600) at a conjugate concentration of 1/1000 to determine the optimal antigen reaction conditions. A serum dilution of 1/100 was employed.

Con A blast controls would therefore be unsuitable for a negative control for OvHV-2 infected lysate from an infected cell-line. No further analysis was performed using OvHV-2 antigen.

5.3.1.2.4. Clinical sera

A blind selection of 20 clinical samples submitted for MCF testing was used for further analysis. All sera were tested in duplicate, at a dilution of 1/100 (as determined in section 5.3.1.3.). Serum K856, employed as a control in the optimization stages of the ELISA, was used as a positive control. The 20 serum samples were examined by both the WC11-ELISA and IFAT. Antibodies against MCFV were detected in seven serum samples by WC11-ELISA. A further three samples reacted negatively in the WC11-ELISA, but appeared positive by IFAT, while one sample reacted positively in the WC11-ELISA, but appeared negative by IFAT. The remaining nine sera gave negative results in both assays (Table 5.2.). The efficiency of the WC11-ELISA for the diagnosis of MCF was tested by determining the relative sensitivity and specificity of the ELISA for the diagnosis of confirmed cases. Using the above set of 20 serum samples and IFAT as the "gold standard" (Table 5.2.), the WC11-ELISA gave a sensitivity of 70% and a specificity of 90%. The Cohen kappa value confirmed moderate agreement between the WC11-ELISA and IFAT when plotted against each other ($k = 0.6$) (Everitt, 1989).

	IFAT			
WC11-ELISA	**Positive**	**Negative**	**Total**	**% Agreement**
				80
Positive (>0.15)	7[a]	1[b]	8	
Negative (<0.15)	3[c]	9[d]	12	
Total	10	10	20	

Table 5.2: Comparison of the relative sensitivity and specificity of the WC11-ELISA compared to IFAT. Relative sensitivity a/a+c = 7/10 = 70%. Specificity d/d+b = 9/10 = 90%.

The four discrepant samples that gave ambiguous results were retested by both tests. One sample tested negative in the WC11-ELISA, changing from positive, and remained positive by IFAT. Two samples tested negative by IFAT, changing from positive, and was confirmed positive by WC11-ELISA (Table 5.3).

Contradictory results were observed for only one animal which reacted positively by IFAT but negatively by the WC11-ELISA (0.071 AU).

Sample number	IFAT	ELISA		Agree	Second test	
		OD	Result		OD	Result
9	Positive	0.352	Positive	Yes	-	-
10	Negative	0.065	Negative	Yes	-	-
11	Positive	0.071	Negative	No	0.001	Unchanged
12	Positive	0.256	Positive	Yes	-	-
13	Negative	0.029	Negative	Yes	-	-
14	Positive	0.058	Negative	No	0.011	IFAT negative
15	Positive	0.200	Positive	Yes	-	-
16	Positive	0.757	Positive	Yes	-	-
17	Negative	0.015	Negative	Yes	-	-
18	Positive	0.12	Positive	Yes	-	-
19	Negative	0.133	Positive	No	0.070	E LISA negative
20	Negative	0.015	Negative	Yes	-	-
21	Positive	1.083	Positive	Yes	-	-
22	Positive	0.587	Positive	Yes	-	-
23	Negative	0.075	Negative	Yes	-	-
24	Positive	0.019	Negative	No	0.016	IFAT negative
25	Negative	0.017	Negative	Yes	-	-
26	Negative	0.027	Negative	Yes	-	-
27	Negative	0.021	Negative	Yes	-	-
28	Negative	0.014	Negative	Yes	-	-

Table 5.3: Blind selection of MCF bovine field serum samples. Samples were analysed by the WC11-ELISA and IFAT (n = 20, 80% agreement).

5.3.1.2.5. Reaction Threshold

Minitab software was used for the statistical analysis of results obtained from the uninfected population of 197 animals to determine the range of negative values and optimise a threshold value for positive reactions. Analysis of the data from the diagnostic tests determined the mean and standard deviation, allowing a 99% confidence threshold value of 0.15 OD units, to be established (Mean plus 3×SD). A reaction threshold of 0.15 for the WC11-ELISA was based on assays, which included a few aberrantly high OD values (Figure 5.7a.), which when retested were all below 0.1 OD. Some of the aberrant values were due to single, aberrant value from a single well. This fact supports the need for repeat testing of such samples or discounting results from aberrant wells for an accurate diagnosis. Of the samples tested, eight samples had an OD value greater than 0.1, of which three had values greater than

0.15. The remaining 189 samples had an OD value of less than 0.1. All samples were retested. After retesting discrepant samples from the first test, a threshold value of 0.1 would have been obtained. It was therefore decided that samples which lie between threshold values of 0.1 and 0.15 should be repeated to obtain a more accurate diagnosis.

All 197 samples were analysed with the CI-ELISA, which detects the presence of immunoglobulins specific for an antigen conserved among MCF viruses (Li *et al.*, 1994, 2001), and association between the tests was analysed. All samples gave negative results in this assay, with inhibition levels less than the established threshold of 25% inhibition (range -72% to 21%). Comparative analysis indicated that the three false positive samples identified by the initial WC11-ELISA were clear negatives in the CI-ELISA (-18 to -33% inhibition). Five serum samples tested had high CI-ELISA values, ranging from 3 to 21% inhibition, while the corresponding WC11-ELISA test results ranged from -0.01 to 0.05 AU. Good correlation was shown between the tests (Figure 5.7b.). The majority of samples formed a population showing low readings in both tests. Dot and box plot analysis of these results are shown in section 5.3.2.

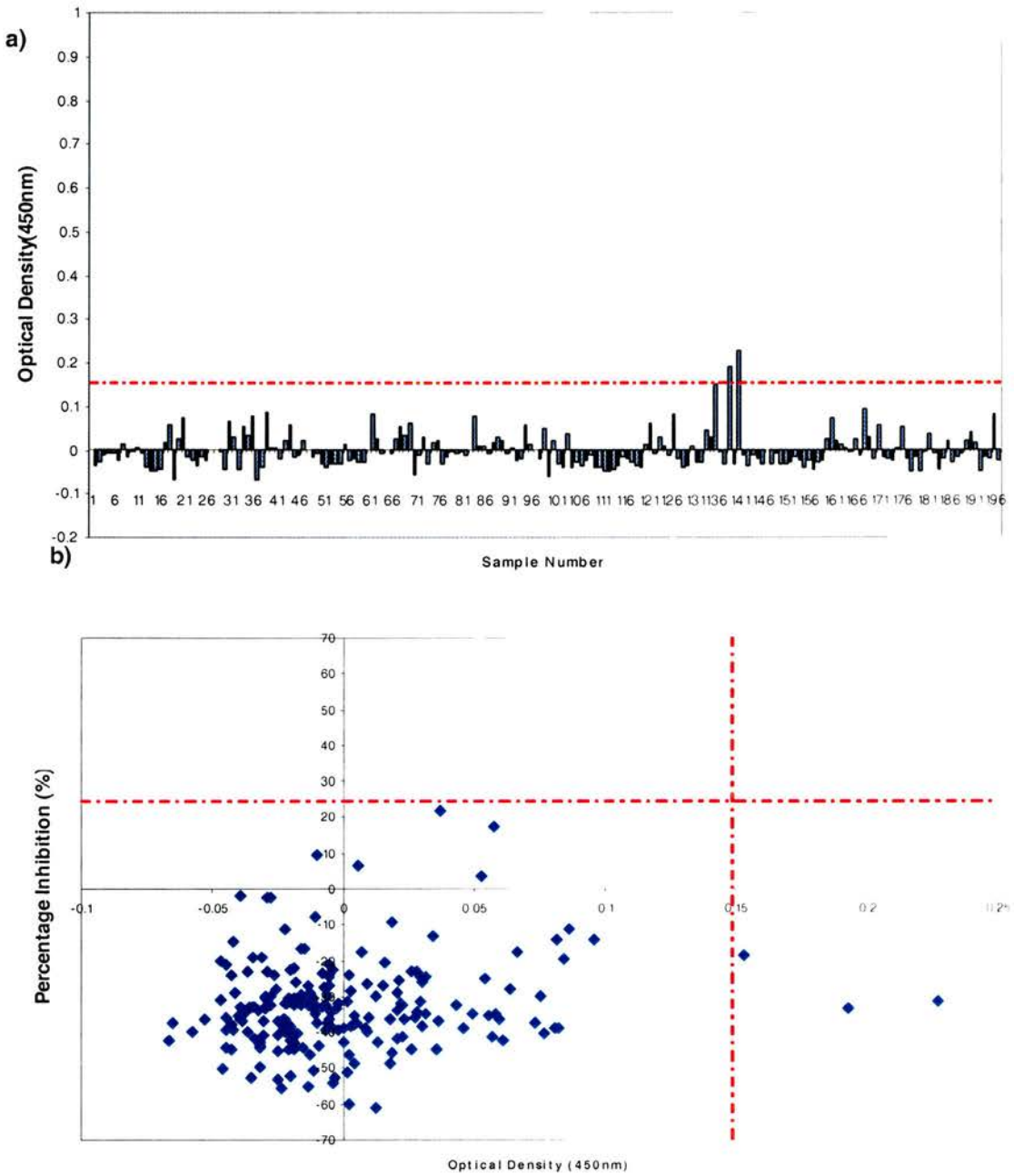


Figure 5.7: Comparison of MCF ELISA tests

a) WC11-ELISA results from the negative control population (n=197). b) Scatter plot of the OD readings (expressed as absorbance at 450nm) for the WC11-ELISA plotted against the percentage inhibition for the CI-ELISA. Cut-offs for each assay are marked with broken lines (WC11-ELISA positive >0.15, CI-ELISA positive >25%).

5.3.2. Comparison of MCFV ELISA tests

Sera from 95 cattle, at a single dilution of 1/100 were examined (in duplicate) by both the CI-ELISA and the WC11-ELISA (Table 5.4.). A single dilution was performed for test samples as was deemed to be the optimal test conditions during optimisation (determined in optimisation of ELISA protocol in section 5.3.1.1). By CI-ELISA, antibodies against the MCFV-specific epitope were detected in twenty-seven serum samples. A further 6 samples reacted negatively in the CI-ELISA, but appeared positive by WC11-ELISA (0.15 threshold). The remaining sixty-two samples gave negative serological results in both assays (Figure 5.8). Of the discrepant samples, five were just below the 25% CI-ELISA reaction threshold value, with sample values ranging from 10-19% inhibition (Inhibition threshold determined by Li *et al.*, 2001). It is unclear why a negative inhibition is caused in the absence of antibodies in the test serum. The occurrence of negative inhibition is not described in either the kit information or the available reference and therefore no explanation can be drawn on the results obtained from the negative values from MCF-negative animals. The test was performed as described in the kit protocol, in accordance with the ELISA developed in Li *et al.*, 2001

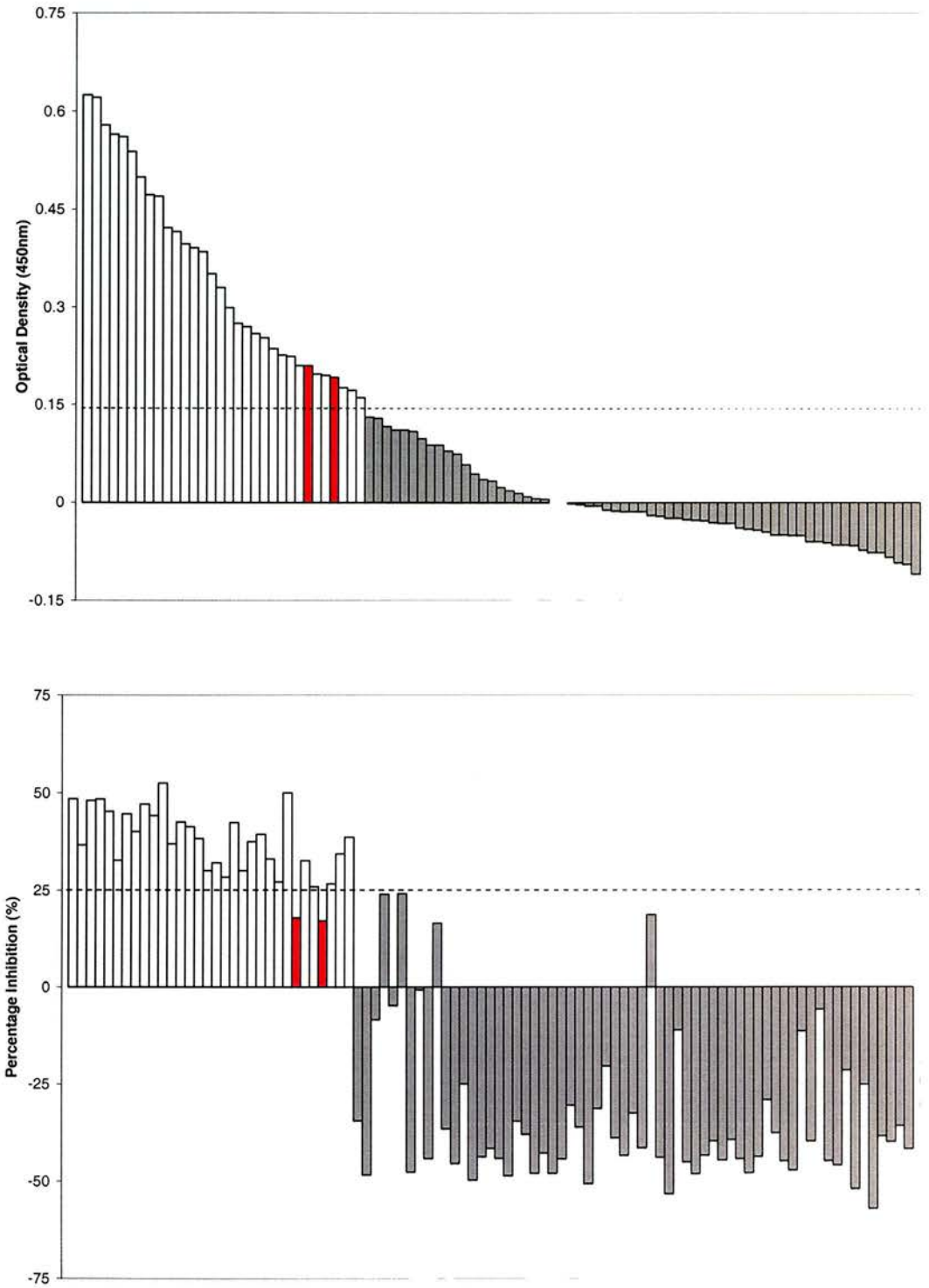
The six discrepant samples were repeated in duplicate with both tests and the mean sample OD calculated (mean positive O.D. minus mean negative O.D.). Three samples subsequently tested positive in the CI-ELISA, remaining positive in the WC11-ELISA. One sample tested negative in the WC11-ELISA, which was confirmed negative by the CI-ELISA. The two remaining samples (samples 48 and 87) were again shown to be negative by CI-ELISA (10% and 22% inhibition) and positive by WC11-ELISA (0.246 and 0.255). Thus, after repeating both tests, contradictory results were observed for only two animals. However for one of these samples, MCF was confirmed by histopathology. Clinical signs and immunofluorescence tests (IFAT) supported this finding. The second CI-ELISA negative/ WC11-ELISA positive serum sample had no histopathology results available but had MCF confirmed by IFAT. No samples that tested negative by ELISA were shown to have MCF confirmed by histopathology or clinical signs.

Sample	CI-ELISA		WC11-ELISA		Sample	CI-ELISA		WC11-ELISA	
	% Inhibition	Result	Optical Density	Result		% Inhibition	Result	Optical Density	Result
1	36.958	Positive	0.397	Positive	32	-44.221	Negative	-0.041	Negative
2	-57.101	Negative	-0.077	Negative	33	-39.594	Negative	-0.032	Negative
3	-5.740	Negative	-0.062	Negative	34	-36.490	Negative	0.074	Negative
4	32.741	Positive	0.538	Positive	35	-41.351	Negative	-0.02	Negative
5	-48.028	Negative	-0.028	Negative	36	-30.457	Negative	-0.003	Negative
6	-21.378	Negative	-0.066	Negative	37	-24.951	Negative	0.044	Negative
7	-39.711	Negative	-0.06	Negative	38	-38.774	Negative	-0.014	Negative
8	-39.828	Negative	-0.093	Negative	39	-42.698	Negative	0	Negative
9	-35.728	Negative	-0.095	Negative	40	-43.284	Negative	-0.031	Negative
10	-50.547	Negative	-0.005	Negative	41	-36.021	Negative	-0.005	Negative
11	-44.807	Negative	-0.051	Negative	42	-43.752	Negative	-0.024	Negative
12	-43.635	Negative	-0.045	Negative	43	-37.544	Negative	-0.05	Negative
13	-47.852	Negative	0.005	Negative	44	24.131	Negative	0.109	Negative
14	-45.920	Negative	-0.065	Negative	45	32.624	Positive	0.197	Positive
15	-29.051	Negative	-0.05	Negative	46	-34.381	Negative	0.131	Negative
16	-0.703	Negative	0.088	Negative	47	-4.686	Negative	0.111	Negative
17	39.360	Positive	0.236	Positive	48	17.103	Negative	0.192	Positive
18	40.121	Positive	0.472	Positive	48a	22.067	Negative	0.145	Negative
19	32.038	Positive	0.299	Positive	49	-47.150	Negative	-0.051	Negative
20	42.405	Positive	0.27	Positive	50	-41.702	Negative	-0.11	Negative
21	45.275	Positive	0.561	Positive	51	10.191	Negative	0.33	Positive
22	-45.041	Negative	-0.027	Negative	51a	30.901	Positive	0.267	Positive
23	-25.068	Negative	-0.077	Negative	52	-47.911	Negative	0	Negative
24	-44.221	Negative	-0.002	Negative	53	25.947	Positive	0.195	Positive
25	-11.363	Negative	-0.06	Negative	54	-48.555	Negative	0.014	Negative
26	-52.011	Negative	-0.073	Negative	55	-44.807	Negative	-0.065	Negative
27	41.351	Positive	0.385	Positive	56	-43.284	Negative	-0.014	Negative
28	30.047	Positive	0.259	Positive	57	-44.514	Negative	-0.032	Negative
29	-8.376	Negative	0.117	Negative	58	27.177	Positive	0.224	Positive
30	-44.104	Negative	0.018	Negative	59	-39.242	Negative	-0.039	Negative
31	44.631	Positive	0.499	Positive	60	-20.324	Negative	-0.013	Negative

Sample	CI-ELISA		WC11-ELISA		Sample	CI-ELISA		WC11-ELISA	
	% Inhibition	Result	Optical Density	Result		% Inhibition	Result	Optical Density	Result
61	38.305	Positive	0.351	Positive	79a	-56.354	Negative	0.098	Negative
62	19.856	Negative	0.21	Positive	80	48.438	Positive	0.565	Positive
62a	50.015	Positive	0.247	Positive	81	37.485	Positive	0.253	Positive
63	38.657	Positive	0.161	Positive	82	-45.392	Negative	0.058	Negative
64	-34.557	Negative	0.009	Negative	83	28.348	Positive	0.275	Positive
65	42.581	Positive	0.391	Positive	84	-49.610	Negative	0.035	Negative
66	0.586	Negative	0.226	Positive	85	-44.104	Negative	0.088	Negative
66a	33.284	Positive	0.586	Positive	86	-31.198	Negative	-0.011	Negative
67	-43.635	Negative	0.033	Negative	87	17.930	Negative	0.21	Positive
68	47.150	Positive	0.47	Positive	87a	10.679	Negative	0.229	Positive
69	34.381	Positive	0.172	Positive	88	-38.441	Negative	-0.084	Negative
70	36.665	Positive	0.621	Positive	89	-32.417	Negative	-0.016	Negative
71	44.162	Positive	0.422	Positive	90	-47.836	Negative	-0.042	Negative
72	48.497	Positive	0.625	Positive	91	-53.215	Negative	-0.024	Negative
73	26.650	Positive	0.176	Positive	92	52.570	Positive	0.416	Positive
74	24.014	Negative	0.111	Negative	93	16.495	Negative	0.079	Negative
75	48.087	Positive	0.579	Positive	94	-11.045	Negative	-0.026	Negative
76	-48.262	Negative	0.129	Negative	95	-37.939	Negative	0.006	Negative
77	18.743	Negative	-0.021	Negative					
78	-41.468	Negative	0.023	Negative					
79	-47.501	Negative	0.151	Positive					

Table 5.4: Field sera from cattle submitted for MCF testing. Sera selected blind from a pool of sera submitted in 2004, examined by both the CI-ELISA and the WC11-ELISA (n = 95). a - represents repeat assay results for discrepant samples

Figure 5.8: Comparison of CI-ELISA and virus-based WC11-ELISA results.
WC11-ELISA analysis of 95 field sera (a), expressed as absorbance reading at 450nm. Un-shaded bars represent positive samples by this test (>0.15, shown in descending order). CI-ELISA analysis of the same samples (b), expressed as percentage inhibition. Un-shaded bars represent positive samples in the WC11-ELISA. Cut-off values for each assay are marked with broken lines (CI-ELISA >25%). Discrepant samples indicated by red bars.


0.75
0.6
0.45
0.3
0.15
0
-0.15
Optical Density (450nm)
75
50
25
0
-25
-50
-75
Percentage Inhibition (%)

To analyse the data from the two ELISA assays, dot and box plots were performed. Dot plots are used to illustrate data distribution, while box plots are used to show the profile of data distribution, central value or median, and variability.

These representations should indicate whether the sample distribution was skewed. Dot plot and box plot analyses showed positive absorbance ranges ranging from 0.625 to 1.51, and negative absorbance ranges ranged from -0.1 to -1.5 OD units in the WC11-ELISA. The CI-ELISA showed positive values ranging from 25 to 55% inhibition and negative values ranged from –60% to 24% inhibition in the CI-ELISA (Figure 5.9a).

Additionally, the box plot results show the distribution of samples in regards to the mean and median points calculated (Figure 5.9b). In the case of the WC11-ELISA, the mean interval point was shown, revealing the negative samples had a restricted distribution, with only a difference of 0.2AU while the positive samples showed a wider distribution, with a difference of 0.45AU. In the case of the CI-ELISA, the distribution was skewed, as results for the majority of the negative sample distribution ranged between -25 and -45% while positive samples ranged between 25 and 55%. The percentage inhibition of individual samples was determined by the tests validation criteria (described in section 2.6.4.2).

5.3.2.1. Evaluation of sensitivity and specificity of the WC11-ELISA

The efficiency of the WC11-ELISA for the diagnosis of MCFV was measured by determining test sensitivity and specificity of the ELISA for the diagnosis of confirmed cases, using the above set of 95 serum samples and the CI-ELISA as the "gold standard" (Table 5.5.). The WC11-ELISA gave a sensitivity of 100% and a specificity of 91% relative to the CI-ELISA. When the CI-ELISA and the WC11-ELISA results were plotted against each other, the Cohen kappa value confirmed an "almost perfect" agreement between the tests ($k = 0.86$) (Everitt, 1989).

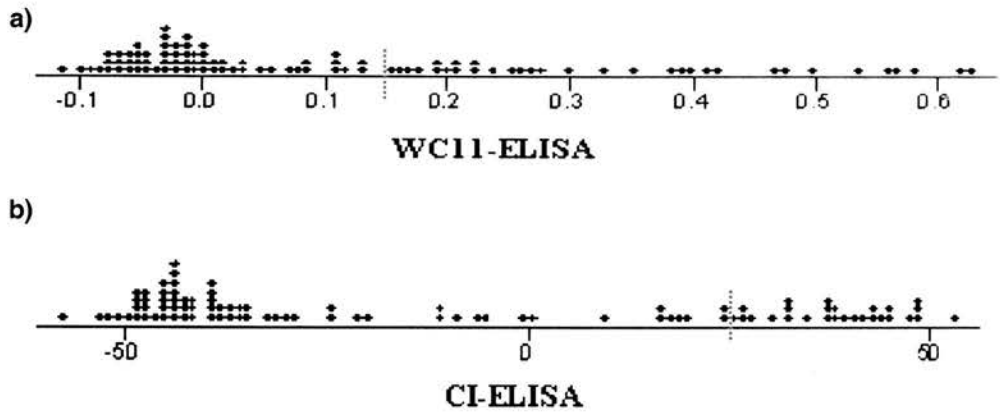


Figure 5.9a: Dot plots distribution. Distribution of absorbance values in the samples analysed by MCF ELISA tests (a) distribution of absorbance ratios in the by the WC11-ELISA and (b) distribution of percentage inhibition by the CI-ELISA. Cut-off values for each assay are marked with broken red lines (CI-ELISA ≥ 25% inhibition, WC11-ELISA OD ≥ 0.15)

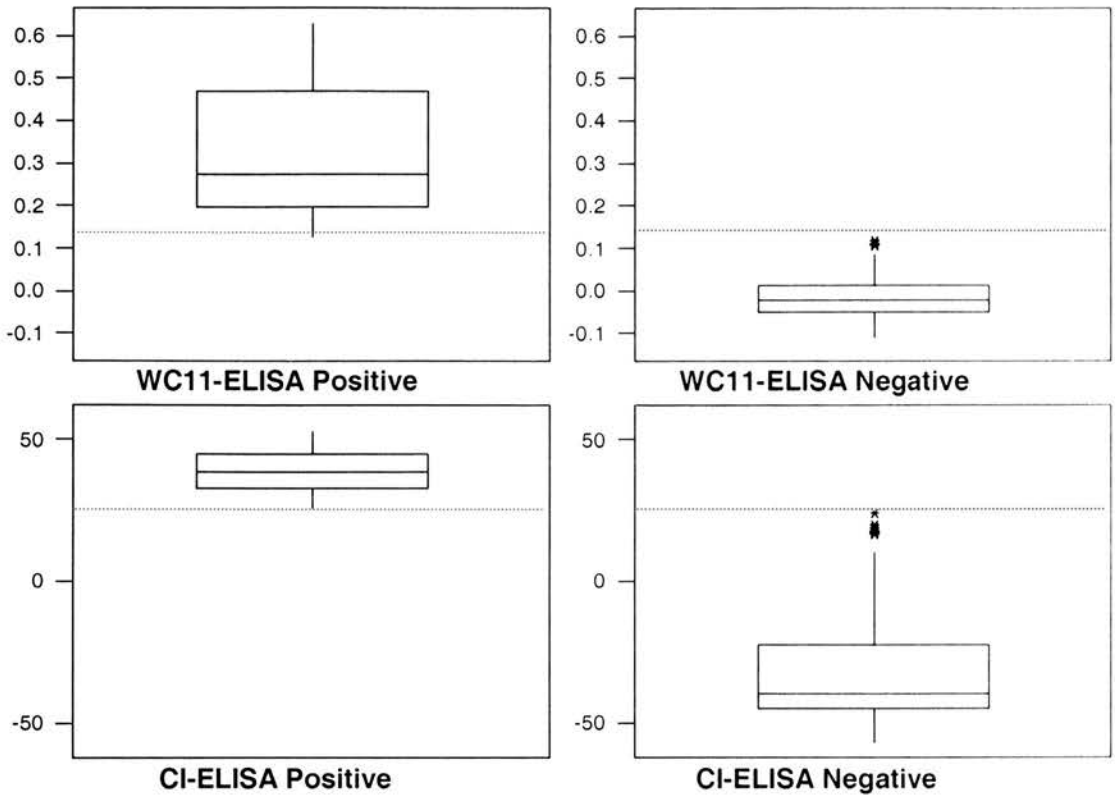


Figure 5.9b: Box plot analysis. Results obtained by the WC11-ELISA and CI-ELISA. Results indicate the variation in sample distributions, in relation to the calculated median (indicated by solid line). Vertical line represents sample range, the box represents the majority of samples and Individual samples (·). Cut-off values for each assay are marked with broken red lines (CI-ELISA ≥ 25% inhibition, WC11-ELISA OD ≥ 0.15)

	CI-ELISA			
	Positive (>25%)	**Negative (<25%)**	**Total**	**% Agreement**
ELISA Result				
Positive (>0.15)	27[a]	6[b]	33	93.7
Negative (<0.15)	0[c]	62[d]	62	
Total	27	68	95	

Table 5.5: Comparison of relative sensitivity and specificity of the WC11-ELISA compared to the CI-ELISA (n = 95). WC11-ELISA shows 100% sensitivity (a/a+c) and 91% specificity (d/d+b) relative to the CI-ELISA.

5.3.2.2. Comparison of MCFV ELISA tests with IFAT

The results obtained from the two ELISA assays were then individually compared to the IFAT results (Figure 5.10), which was used as the "gold standard". Antibodies against MCFV were detected in 44 serum samples by IFAT. Fifty samples gave negative serological results in all assays The WC11 ELISA gave a sensitivity of 65% and a specificity of 94% (Table 5.6.) and showed an overall agreement of 81% (k = 0.61) when compared with IFAT. The CI-ELISA gave a sensitivity of 63% and a specificity of 94% relative to IFAT (Table 5.7.) and showed an overall agreement of 80% (k = 0.59).

Antibodies against MCFV were detected in twenty-eight serum samples by WC11-ELISA. Fifteen samples reacted negatively in the WC11-ELISA, but appeared positive by IFAT (Table 5.6). Antibodies against the MCFV-specific epitope were detected in twenty-seven serum samples by CI-ELISA. Sixteen samples reacted negatively in the CI-ELISA, but appeared positive by IFAT. The results for both ELISA tests showed the same three samples were ELISA positive but IFAT negative.

	IFAT			
	Positive	**Negative**	**Total**	**% Agreement**
ELISA Result				
Positive (>0.15)	28[a]	3[b]	31	81
Negative (<0.15)	15[c]	49[d]	64	
Total	43	52	95	

Table 5.6: Comparison of relative sensitivity and specificity of the WC11-ELISA compared to IFAT. WC11-ELISA showed 65% sensitivity (a/a+c) and 94% specificity (d/d+b), relative to IFAT.

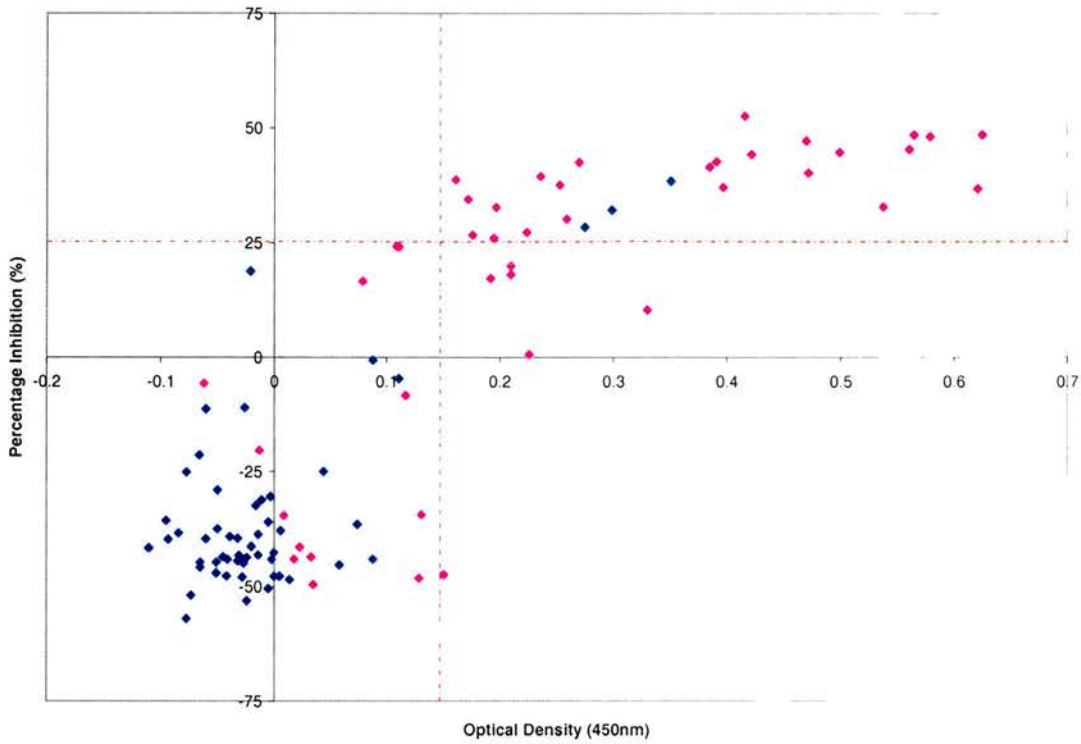


Figure 5.10: Comparison of CI-ELISA, WC11-ELISA and IFAT results.

Scatter plot of the optical density value (expressed as absorbance reading at 450nm) for the WC11-ELISA plotted against the percentage inhibition for the CI-ELISA (highlighted blue). Samples positive by IFAT are highlighted in pink. Cut-off value for each assay (CI-ELISA >25% WC11-ELISA >0.15) marked with broken red lines.

	IFAT			
	Positive	**Negative**	**Total**	**% Agreement**
CI-ELISA Result				
Positive (>0.1)	27[a]	3[b]	30	80
Negative (<0.1)	16[c]	49[d]	65	
Total	43	52	95	

Table 5.7: Comparison of relative sensitivity and specificity of the CI-ELISA compared to IFAT. CI-ELISA showed 63% sensitivity (a/a+c) and 94% specificity (d/d+b), relative to IFAT.

5.3.2.3. ELISA Specificity

In order to investigate potential cross-reactivity in the WC11-ELISA, 95 diagnostic serum samples positive for bovine herpesvirus-4 (BoHV-4), infectious bovine rhinotracheitis virus (BoHV-1/IBR) and bovine viral diarrhoea virus (BVDV) were tested for reactivity in the WC11-ELISA. The specificity of the WC11-ELISA for antibodies to antigens of alcelaphine herpesvirus-1 was demonstrated by the absence of a positive reaction in all cases. Serum samples yielded low OD readings of less than the established positive reaction threshold of 0.15, with values ranging from 0 to -0.026. When tested by CI-ELISA, serum samples yielded inhibition levels considerably less than the established reaction threshold of 25% inhibition, ranging from -35 to -40% inhibition. Total agreement was therefore shown between the WC11-ELISA and CI-ELISA.

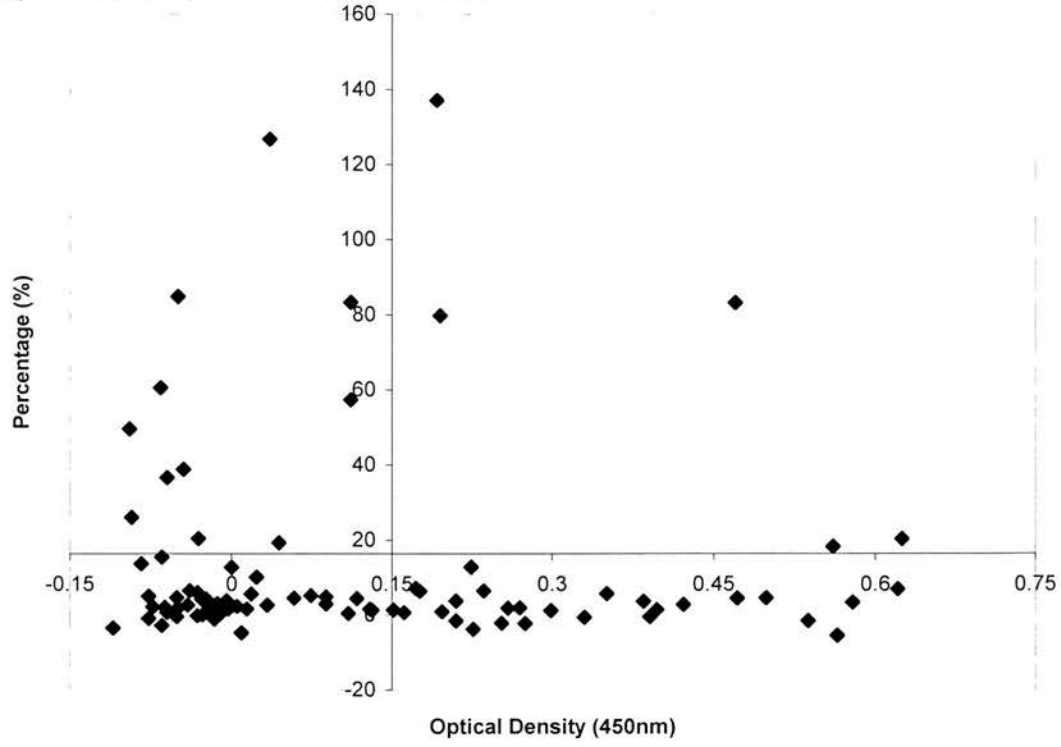
5.3.2.4. Bovine herpesvirus-4 cross-reactivity

Potential cross-reactivity of the WC11-ELISA with bovine herpesvirus-4 was also analysed by testing the ninety-five serum samples previously tested (described in section 5.2.5) by a direct BoHV-4 ELISA (Bio-X Diagnostics). BoHV-4, like AlHV-1 is a gammaherpesvirus and was specifically studied since it has been shown to cross react in tests such as IFAT (Li *et al.*, 1994; Dewals *et al.*, 2005).

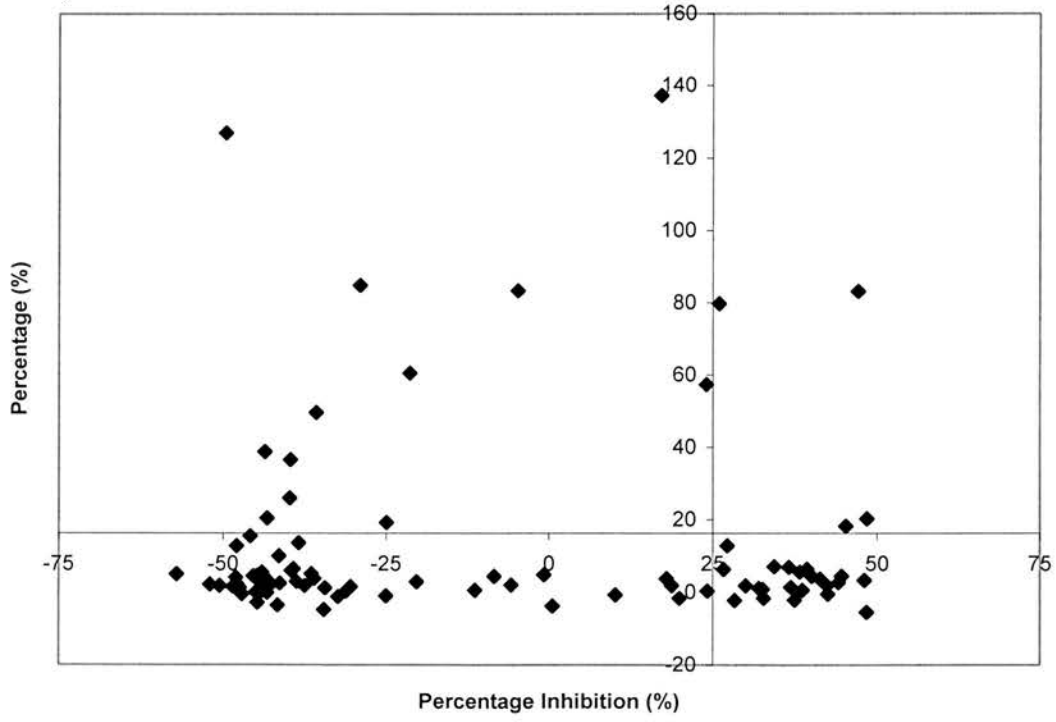
Of the sera tested, sixteen samples were positive by the BoHV-4 ELISA. Of these samples, the WC11-ELISA determined five BoHV-4 positive samples as MCF positive (Figure 5.11a.), four of which were also positive by CI-ELISA (Figure 5.11b.). All five samples were found positive for MCF by IFAT. No other BoHV-4 positive samples were positive for MCF by IFAT. In the case of the single WC11-ELISA positive (0.192 OD value)/CI-ELISA negative (17% inhibition) and BoHV-4

Figure 5.11: Correlation of reactivity of ruminant sera by the MCF and BoHV-4 ELISA assays. a) Correlation of reactivity of ruminant sera by the MCF WC11-ELISA and BoHV-4 ELISA assay (n= 95, R^2 = 0.0004). b) Correlation of reactivity of ruminant sera by the MCF CI-ELISA and BoHV-4 ELISA assay (n= 95, R^2 = 0.0021). Cut-off value for assay (BoHV-4 >16.35, CI-ELISA >25% and virus-based ELISA >0.15) marked by axis intercept.

a) WC11-ELISA Vs BoHV-4 ELISA



B) CI-ELISA Vs BoHV-4 ELISA



positive sample (137.15%), MCF was confirmed by both IFAT and by histopathogical findings.

5.3.3. Investigation of MCF Outbreaks

5.3.3.1. Case Study One

In one beef farm, seven cows out of seventy-eight became ill and subsequently died over a 6 month period. All of the affected animals showed characteristic signs of MCF, and were all IFAT-positive, confirming MCF. Sera from the remaining seventy animals in the herd were submitted for experimental testing of MCF using the IFAT assay, CI-ELISA and WC11-ELISA (Table 5.8) and the results were compared.

There was good correlation between all tests for most serum samples (Figure 5.12). All seventy sera reacted negatively in the CI-ELISA. Fourteen samples were positive by IFAT, one of which was positive by the WC11-ELISA. None of the cattle analysed developed clinical signs of MCF. Fourteen IFAT-positive serum samples were tested for BoHV-4 cross-reactivity in both the IFAT and the WC11-ELISA, all the samples were BoHV-4 negative by the BoHV-4 ELISA.

5.3.3.2. Case Study Two

On a beef suckler farm, four 14-18 month old steers showed signs of MCF and subsequently died over a period of three weeks. A further four animals were later affected and subsequently died. No classical MCF head and eye lesions were observed although clinical signs included pyrexia and an increased respiratory rate.

Animal	Serology Test			Animal	Serology Test		
	IFAT	CI-ELISA (percentage Inhibition)	WC11-ELISA (Optical Density 450nm)		IFAT	CI-ELISA (percentage Inhibition)	WC11-ELISA (Optical Density 450nm)
1	**Positive**	-10.5326	-0.008	**26**	Negative	-46.3578	0.01
2	Negative	-36.9716	0.006	**27**	**Positive**	-37.1149	0.022
3	Negative	-8.81299	0.129	**28**	Negative	-29.1617	-0.011
4	Negative	-26.3673	0.023	**29**	Negative	-31.8128	-0.002
5	Negative	-40.6974	-0.018	**30**	Negative	-33.6757	-0.014
6	Negative	-31.1679	-0.002	**31**	Negative	-18.4141	0.018
7	Negative	-35.1087	0.011	**32**	Negative	-22.5699	-0.042
8	Negative	-39.6943	0.032	**33**	**Positive**	-37.5448	-0.031
9	Negative	-23.8596	0.031	**34**	Negative	-27.7287	-0.047
10	Negative	-29.0184	0.022	**35**	Negative	-38.6912	-0.025
11	Negative	-44.2083	-0.069	**36**	Negative	10.6759	-0.021
12	Negative	-35.7535	-0.03	**37**	Negative	-16.3363	-0.033
13	Negative	-28.7318	-0.054	**38**	Negative	-84.9773	-0.049
14	Negative	-25.1493	-0.034	**39**	Negative	-14.6167	-0.015
15	Negative	-27.1555	-0.074	**40**	**Positive**	-10.6043	-0.007
16	Negative	-31.3829	-0.022	**41**	**Positive**	-22.9281	**0.454**
17	Negative	-4.15572	0.009	**42**	**Positive**	-38.6912	0.106
18	Negative	-36.2551	-0.052	**43**	**Positive**	-46.716	0.131
19	Negative	-40.1958	-0.034	**44**	Negative	-32.6009	0.008
20	Negative	-7.66659	0.022	**45**	Negative	-33.4607	-0.009
21	Negative	-26.5823	-0.019	**46**	Negative	-33.604	0.016
22	Negative	-31.0963	-0.018	**47**	Negative	-6.23358	0.127
23	Negative	-12.7538	-0.013	**48**	Negative	-41.9871	0.041
24	Negative	-22.9998	0.04	**49**	Negative	-25.4359	-0.011
25	**Positive**	-35.3953	-0.002	**50**	Negative	-40.984	-0.027

Animal	Serology Test			Animal	Serology Test		
	IFAT	CI-ELISA (percentage Inhibition)	WC11-ELISA (Optical Density 450nm)		IFAT	CI-ELISA (percentage Inhibition)	WC11-ELISA (Optical Density 450nm)
51	Negative	-24.2895	-0.045	**62**	Negative	-44.9248	0.07
53	Negative	-36.3984	-0.049	**63**	Negative	-43.7067	0.09
54	Negative	-46.4294	-0.027	**64**	**Positive**	-42.9186	0.106
55	Negative	-32.386	-0.025	**65**	**Positive**	-41.3422	0.034
56	Negative	-27.7287	-0.037	**66**	**Positive**	-41.8438	0.016
57	**Positive**	-39.4793	-0.047	**67**	**Positive**	-22.2833	0.02
58	Negative	-25.0776	-0.031	**68**	Negative	-31.4545	-0.014
59	Negative	-26.5106	-0.006	**69**	Negative	-33.1025	-0.021
60	**Positive**	-28.3735	-0.055	**70**	Negative	-95.7249	-0.036
61	Negative	-42.0588	0.008				

Table 5.8: Comparison of three MCF diagnostic tests. To test assay sensitivity, the three serological assays (IFAT, CI-ELISA and WC11-ELISA) were analysed for the diagnosis of MCF in surviving animals from an MCF outbreak (n = 70). Positive samples highlighted in red.

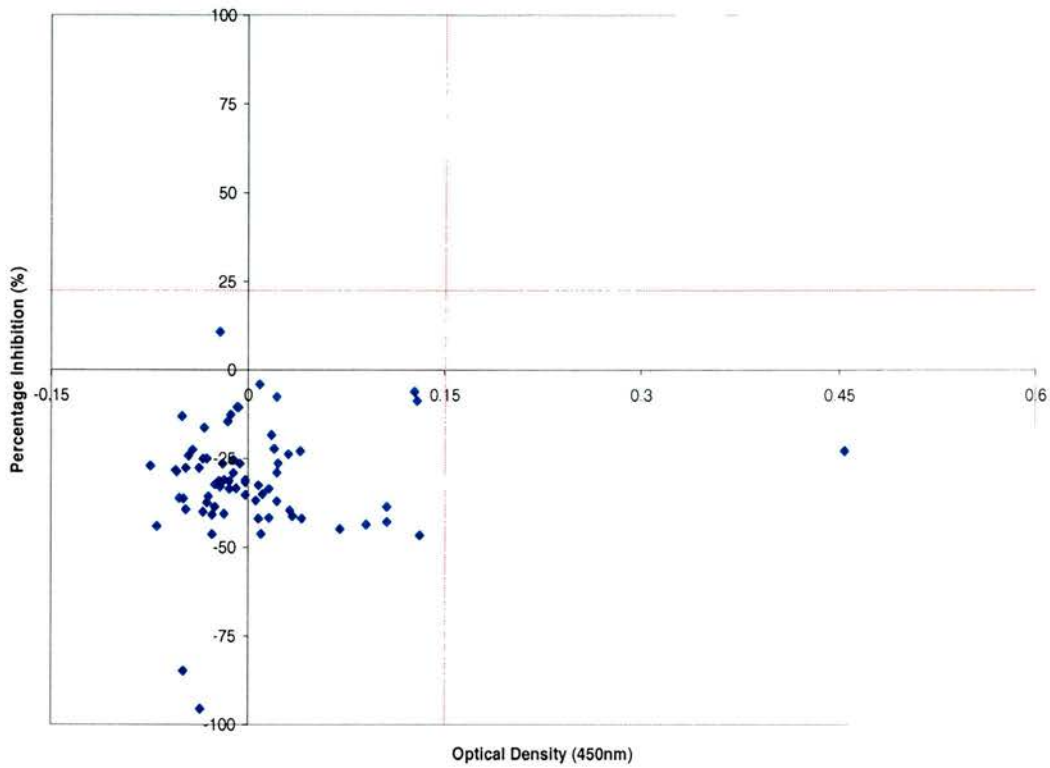


Figure 5.12: ELISA analysis of MCF outbreak (n = 70). Scatter plot of the OD readings (expressed as absorbance at 450nm) for the WC11-ELISA plotted against the percentage inhibition for the CI-ELISA. Cut-offs for each assay are marked with broken lines (WC11-ELISA positive >0.15, CI-ELISA positive >25%).

Necropsy analysis identified extensive ulcerations of abomasal mucosa, froth and fibrous ingesta in tracheal and bronchial lumen, pulmonary oedema, generalised lymphoadenopathy, haemorrhages on the bladder and meningeal opacity.

Histopathology results for two of the four affected animals showed inflammatory changes in many tissues suggestive of MCF, and OvHV-2 was confirmed by analysis of spleen tissues by PCR. Blood samples from the surviving animals in the group (n = 10) were tested for MCF using IFAT, PCR, CI-ELISA and WC11-ELISA (n = 10) (IFAT performed by VSU, & PCR assay performed by G. Russell, Moredun Research institute). There was good correlation between test systems for most specimens. One animal which was shown to be positive for MCF by CI-ELISA, WC11-ELISA and PCR, subsequently developed clinical MCF. The remaining nine samples reacted negatively. All samples gave negative serological results by IFAT.

5.3.3.3. Case Study Three

On a beef suckler farm, seven animals showed signs of MCF and subsequently died over a period of 5 months. Classical MCF symptoms were described, including corneal oedema, ocular and nasal discharge and high body temperatures (≥40°C). No histopathology results were available, however, MCF was confirmed in the 7 animals by IFAT.

Bloods from the surviving animals in the group (n = 58) were submitted for experimental testing of MCF using IFAT, PCR and WC11-ELISA (IFAT performed by VSU, & PCR assay performed by D. Grant, MCF group, Moredun Research Institute) and the results compared. There was good correlation between test systems for most samples. One animal was positive by WC11-ELISA and OvHV-2 real-time PCR (described by Baxter *et al.*, 1993), while the remaining fifty-seven samples reacted negatively. All samples gave negative serological results by IFAT.

5.4. Discussion

The aim of this research was the development of a reliable, efficient virus-based ELISA assay for the detection of antibodies against malignant catarrhal fever virus in serum and to evaluate this assay against established diagnostic assays including the standard indirect immunofluorescence assay and the competitive-inhibition ELISA. A validated assay may identify animals as either positive or negative for an antibody, and can accurately predict the infectious status of an individual using a predetermined degree of statistical error (Office International des Epizooties, 2003). Initial stages required in the development of a new diagnostic assay, such as an ELISA, consist of extensive studies into the feasibility of protocols, analysis and optimisation of reagents (Office International des Epizooties, 2003). The variables which were considered prior to sampling field serum included; dilutions of MCFV antigen, serum samples (antibody) and conjugated antibody dilutions. The determination of these variables allowed optimum reaction conditions to be established preceding serum sample testing.

Crude lysate antigens from AlHV-1 strains C500 and WC11 were prepared and tested with MCF serum samples from cattle with clinical symptoms indicative of potential MCF infection. The AlHV-1 strain WC11 showed 100% correlation with IFAT while the AlHV-1 strain C500 ELISA showed one discrepancy. An OvHV-2 lysate-based ELISA was also tested. This gave optical density values that were greater for the control antigen than the infected cell-lysate. The AlHV-1 strain WC11 ELISA antigen was therefore selected for further analysis.

Good positive agreement between the magnitude of reaction in the WC11-IFAT and ELISA was demonstrated. Both tests were able to detect animals with MCF with a moderate degree of accuracy and agreement ($k = 0.6$). Parallel testing of 20 serum samples, including cattle infected with ovine herpesvirus-2, showed 80% concordance between IFAT and WC11-ELISA. The number of seropositive cattle detected by IFAT was greater than the number detected by WC11-ELISA. Although this suggests the WC11-ELISA was less efficient in its accurate detection of MCF, giving false negative results, the discrepant samples were repeated by both tests. After re-testing, only one sample still gave contradictory results reacting positively by IFAT but negatively by the WC11-ELISA. The fact that results of multiple IFAT tests changed from positive to negative suggests it may not be an ideal standard against which to test the direct ELISA. Differences in results may indicate a number of factors, such as sample deterioration, operator error or changes in reagents

employed. One result of the ELISA test also changed from a low positive to a negative. This may be a result of the lack of positive threshold to accurately discriminate between positive and negative samples. Therefore a threshold should be determined to ensure test results are repeatedly correct, in addition to analysing the difference between a high negative and a low positive.

Preliminary studies found a high level of concordance between the standard IFAT test and the WC11-ELISA. The data obtained by the described ELISA indicates its value as a simple, economical, rapid serological assay for the detection of antibodies against MCFV. To complete the establishment and initial validation of this assay, serum samples submitted for MCF testing were collected and analysed in a parallel study to allow a comparison of IFAT, WC11-ELISA and the commercially available MCF ELISA (CI-ELISA). Good agreement between the magnitude of reaction in the CI-ELISA and the WC11-ELISA was demonstrated. Both tests were able to detect animals with MCF to a high degree of accuracy and agreement (k = 0.86). Parallel testing of 95 serum samples from cattle with suspected MCF revealed over 93% concordance between CI-ELISA and WC11-ELISA. Two more animals were tested seropositive by WC11-ELISA than CI-ELISA, showing 100% sensitivity and 91% specificity relative to diagnosis by CI-ELISA. Although this suggests the WC11-ELISA is less specific in its accurate detection of MCF, one sample that was CI-ELISA negative/ WC11-ELISA positive, was also IFAT positive and showed clinical signs of MCF, while a further sample had MCF confirmed by histopathological evidence. The result of the CI-ELISA for this case was narrowly under the positive threshold (22% inhibition). This would suggest that the WC11-ELISA may be more sensitive than the CI-ELISA in its detection of antibodies against MCF viruses. This difference may be related to the fact that CI-ELISA relies on inhibition of binding of a monoclonal antibody and hence focuses on a single epitope whereas the WC11-ELISA relies on a polyclonal response recognising multiple epitopes and consequently may be more robust where the infected animal has limited or low antibody production.

The CI-ELISA showed a skewed distribution of the results for the 95 samples tested. Statistical analysis showed that this distribution was not a direct result of the calculation used to determine percentage. Li *et al.*, (1994) stated that the concentration of monoclonal antibody was less critical than antigen concentration to the sensitivity of the assay. The assay sensitivity may be a result of a low binding affinity, where a limited amount of antigen means that little blocking antibody is

necessary to produce a clear inhibitory result. The test reaction conditions used may explain the skewed distribution of results.

The positive threshold limit of 0.15 for the WC11-ELISA was based on assays that included a few aberrantly high OD values in individual wells. By discounting these discrepant samples, a threshold of 0.1 would have been obtained. Consequently, any samples that gave readings of between 0.1 and 0.15 were recorded as inconclusive and were re-tested. Of the six samples that were discrepant between the WC11-ELISA and CI-ELISA, five were close to the CI-ELISA positive threshold value (10-19% inhibition). Re-testing of these samples showed three of these five tested positive by CI-ELISA. This indicates that routine use of duplicate CI-ELISA tests may improve test accuracy.

Parallel testing of the 95 serum samples by both ELISA assays and by IFAT revealed over 86% concordance between the WC11-ELISA and IFAT assays, and 80% concordance between the CI-ELISA and IFAT. The number of seropositive cattle detected by IFAT was greater than the number detected by either ELISA assay, showing 73% sensitivity and 98% relative specificity when compared to the WC11-ELISA, and 59% sensitivity and 98% relative specificity when compared to the CI-ELISA. These findings support the view that the number of seropositive cattle detected by IFAT was always equal to or greater than the number detected by the two ELISA tests. IFAT has been reported to show cross-reactivity with other bovine herpesviruses (Wan *et al.*, 1988). However, in this study BoHV-4, BoHV-1 and BVDV-specific sera did not cross-react with AlHV-1 infected cells by IFAT.

Other authors have also noted possible serological cross-reactivity between MCFV and other herpesviruses (Li *et al.*, 1994; Dubuisson *et al.*, 1989; Li *et al.*, 1995). Studies by Osorio *et* al., (1985) showed BoHV-1 and BoHV-2 did not serologically cross-react with other bovine herpesviruses, although they are found to cross-react with each other. Dewals *et al.*, (2005) showed that BoHV-4 specific antiserum stained antigens expressed in the nucleus of AlHV-1 infected cells. They also indicated that this relationship was unidirectional as AlHV-1 specific serum showed no detectable staining of BoHV-4 infected cells. The lack of cross-reactivity of BoHV-4 serum in the WC11-ELISA may therefore reflect the loss of cross-reactive, possibly nuclear, AlHV-1 antigens during preparation/extraction of the ELISA antigen.

In this study, of the samples tested by the BoHV-4 ELISA, sixteen were BoHV-4 positive. Of these serum samples, the number of BoHV-4/MCF seropositive

cattle detected by the WC11-ELISA (n = 5) was greater than the number detected by CI-ELISA (n = 4). In the case of the single WC11-ELISA positive (0.19)/CI-ELISA negative (17%) and BoHV-4 positive sample, MCF was confirmed by histopathogical findings. All five samples were additionally found positive for MCF by IFAT. These results highlight problems regarding the cross-reactivity between MCF and BoHV-4. It is possible that the five animals diagnosed as MCF positive and BoHV-4 positive have had previous exposure to both viruses and therefore show antibodies to both. It could alternatively suggest that some animals may develop antibodies to cross-reacting epitopes. If true, this appears to occur rarely due to the small number of BoHV-4 animals which are diagnosed MCF positive.

The specificity of the ELISA for antibodies to antigens of alcelaphine herpesvirus-1 was demonstrated by the absence of a positive reaction to hyperimmune serum samples specific for other bovine viruses including the alphaherpesvirus BoHV-1 and the gammaherpesvirus BoHV-4. Hyperimmune serum for bovine viral diarrhoea virus (BVDV) also tested negative by the WC11-ELISA. Although BVDV is a member of the pestivirus family and there is no serological cross-reactivity between these virus families, it was important to demonstrate that a differential diagnosis can be made since the clinical signs can be very similar.

MCF typically emerges sporadically, with a small number of affected animals (Brenner *et al.*, 2002) but outbreaks where several animals in a herd become affected have been reported and can occur on the same farm in consecutive years. There have been many reports of outbreaks of multiple cases (Hamilton., 1990; Collery and Foley., 1996; Yus *et al.*, 1999; Brenner *et al.*, 2002; Dabak and Bulut, 2003; Otter, Pow & Reid., 2002; Abu Elzein *et al.*, 2003) in contrast to the more characteristically sporadic disease, the reason for which remains unknown.

As an additional validation step, samples from herds where MCF outbreaks were reported were tested to assess whether animals showed an antibody response before the onset of clinical signs and to allow a comparison to be made concerning the current diagnostic tools. This was shown in the second outbreak analysed where one of the ten animals tested showed an antibody response by both ELISA tests and later developed clinical MCF. This suggests that the WC11-ELISA can detect MCF before the onset of clinical signs and this could be very beneficial when monitoring outbreaks. Although OvHV-2 infections in cattle commonly result in the development of clinical signs within weeks, animals have been reported where no clinical signs develop, and indicate the possibility of silent infections within outbreak

herds (Otter *et al.*, 2002). The ELISA would therefore prove beneficial for the large-scale testing of animals present in an infected herd, or just for routine surveillance.

The first outbreak involved a herd consisting of seventy-eight animals, where seven animals were affected and subsequently died. Heparinised blood samples from the surviving seventy-one in-contact animals in this group were examined for serological evidence of MCF. Samples were tested by IFAT, CI-ELISA and WC11-ELISA. All were negative by the CI-ELISA, while one sample was demonstrated as a low positive by the WC11-ELISA. By IFAT, fourteen in-contact animals from the group were positive, including the sample positive by WC11-ELISA, none of which developed clinical disease. Additionally, all IFAT positive samples tested gave negative serological results by BoHV-4 ELISA. These findings support the earlier statement that the number of seropositive cattle detected by IFAT was always greater than the number detected by other diagnostic methods (Li *et al.*, 1994). This may be partly due to the test being subject to visual interpretation and partly to cross-reactivity with other bovine herpesviruses, although cross-reactivity to BoHV-4 was ruled out in this case study.

A second outbreak of MCF resulted in the deaths of eight cattle in a group of beef suckler steers. Heparinised blood samples taken from ten in-contact surviving animals were analysed. The CI-ELISA, WC11-ELISA and PCR results all showed 100% agreement, revealing the infection in one other animal, which later developed clinical disease. All samples were negative by IFAT. This is the only case where IFAT did not detect ELISA positive serum. The fact that this sample was taken before symptoms developed may be significant, suggesting there may be a difference in serum titre pre-/post development of clinical signs. The delay between sampling and the onset of clinical signs in these cases is unknown. Knowing the period of time between clinical signs and sampling could result in differences in antibody responses and ultimately diagnosis. It would therefore be advised to test animals at regular intervals; this however would be unlikely as routinely, testing would only be initiated on suspicion of MCF disease.

Preliminary studies have found a high level of concordance between the commercially available CI-ELISA and the WC11-ELISA. The data obtained by the described ELISA indicates its value as an economical, rapid and specific serological assay for the detection of antibodies against MCFV. The simplicity of the WC11-ELISA and its lack of cross-reactivity make this assay a suitable test for routine

MCFV diagnosis and epidemiology in laboratories world-wide. Where appropriate secondary reagents and conjugates are available, analysis of sera from exotic species that carry or are susceptible to MCFV may also be possible.

Chapter Six
Validation Studies

6.1 Introduction

The recent advances in molecular and serological diagnostic assays for the detection of malignant catarrhal fever have provided powerful tools for investigating the pathogenesis and epidemiology of MCF (Hsu *et al.*, 1990: Li *et al.*, 2000). Histopathological examination remains the recognised diagnostic procedure to confirm MCF infection. However, since PCR assays have proved sensitive and specific, such methods may be established as a new "gold standard" for diagnosis following validation studies between laboratory tests. The sensitivity of the PCR methods has allowed the monitoring of MCF infection in carrier and susceptible animals, enabling the pathogenesis of the viral disease to be investigated (Baxter *et al.*, 1993).

There are, however, advantages offered by serological testing. Serology offers a means of testing large numbers rapidly, providing an increased understanding regarding epidemiological aspects of MCF. Therefore large groups of animals at risk of developing MCF in highly susceptible species such as deer and bison can be monitored (O'Toole *et al.*, 2002). By routine testing using serological methods, control measures may ultimately be established, whereby no infected animals may be brought into a non-infected flock or herd (Li *et al.*, 1999). A second advantage to using serological tests is the demonstration that many animals which have had past infections may be serologically positive but PCR negative (Colins *et al.*, 2000; O'Toole *et al.*, 2002). Studies investigating OvHV-2 infections by PCR and CI-ELISA (O'Toole *et al.*, 2002; Powers *et al.*, 2005) speculated that animals which were serologically positive but DNA negative may represent a latent form of infection. During latency, viral load in blood cells may be below detectable levels by PCR.

Serological tests offer economical, rapid and specific methods for the detection of antibodies against MCFV. The simplicity of serological assays such as the CI-ELISA and WC11-ELISA (developed in chapter five) and lack of cross-reactivity, offer a reliable, low cost method for routine MCFV diagnosis and epidemiological studies and long-term monitoring.

On preliminary characterisation of the CI-ELISA, a high level of agreement was found between the ELISA and IFAT (Li *et al.*, 1995). No samples negative by IFAT were positive by ELISA suggesting that cross-reactivity may be responsible for the greater number of IFAT positive results (Li *et al.*, 1995; Li *et al.*, 1996). The CI-ELISA was also compared to the OvHV-2 PCR in parallel testing and revealed a

substantial agreement between the tests. The difference in the results was thought to be due to a lower production of antibodies in some cases. PCR was found to be the most reliable test for the determination of acute clinical MCF cases when compared to serological tests, although both antibody assays proved beneficial in the determination of MCF infected animals. A study by Muller-Doblies *et al.*, (1998) assessing the OvHV-2 PCR and CI-ELISA in comparison with histopathological results, also found that PCR was the method of choice for diagnosis. Of the cases examined by PCR, ELISA and histopathology, two cases inconclusive by pathology were confirmed positive by PCR, while the CI-ELISA was less sensitive than both histopathology and PCR (Muller-Doblies *et al.*, 1998).

This chapter describes preliminary validation studies of the WC11 based ELISA to detect anti-MCF virus antibodies and compares results obtained with established serological methods. Firstly, a longitudinal study of cattle experimentally immunised with AlHV-1 was analysed. Animals were sampled at weekly intervals after inoculation and serum samples were tested using a serum neutralisation assay in comparison with the WC11-ELISA. A second study into the validation of the WC11-ELISA was performed. Bovine samples, submitted for MCF diagnosis, were tested by three serological assays; CI-ELISA, IFAT and WC11 ELISA; the OvHV-2 specific real-time diagnostic PCR and histopathology/veterinary diagnosis. Samples were additionally tested using a BoHV-4 ELISA (BioX Diagnostics) to examine the issue of cross-reactivity between the gammaherpesviruses.

6.2 Materials and Methods

6.2.1. DNA preparation

Genomic DNA was extracted from fresh tissues and blood using the Qiagen DNeasy tissue kit, in accordance with the manufacturer's directions (Qiagen DNeasy tissue handbook, 03/2004). DNA was quantified by UV absorbance at 260nm (Cecil Spectrophotometer: CE2041 2000 series).

6.2.2. Serological Methods

Serological methods (WC11-ELISA, CI-ELISA) and reagents were used as previously described in chapter 5.

6.3. Results

6.3.1. Longitudinal Study of Experimentally Infected Animals

Serum samples from animals intramuscularly vaccinated with AlHV-1 strain C500 (described in section 2.1.2) collected weekly were monitored by the established virus neutralisation assay (Mushi and Plowright, 1979; Reid *et al.*, 1975) and the newly developed WC11-ELISA (Section 5.2.1-5.2.2). These animals were vaccinated in order to induce an immune response to a non-pathogenic, attenuated MCF virus and were later challenged with a pathogenic viral strain to elicit infection. Tests were performed to analyse the animals' immune response to the virus.

Initial findings suggested that the virus neutralisation assay was more sensitive than the ELISA (Table 6.1), detecting an immune response 7-14 days earlier. This may be a direct result of the ELISA employing a 1/100 test serum dilution, rather than the serum titration method employed by neutralisation. The virus neutralisation assays showed high and persistent levels of virus-neutralising antibodies, particularly in the calves intranasally boosted with AlHV-1 (vaccination trial performed by I. Campbell and G.Russell as described in section 2.1.2, Figures 6.1 and 6.2). Variation was observed between weekly samples which are thought to be a result of experimental assay variation rather than showing evidence of cyclic patterns in the neutralisation responses.

The ELISA mirrored the general trend in antibody response for each animal, and showed less variation than the neutralisation assay. One animal from each group developed clinical MCF following challenge with virulent AlHV-1 (day 154 post-vaccination). These animals were seropositive until death from clinical MCF at twenty-three (200819) and forty-three (400251) days post-challenge (Figures 6.4 and 6.1 respectively). Typical histopathological lesions were shown in the lymph nodes and major organs including liver and intestine. Interestingly, the antigen/chitosan boosted animal which developed clinical MCF (200819) showed a decline in antibody response subsequent to challenge (Figure 6.3). This may suggest an effect of acute MCF on the immune system. This decline in the immune response was not found in the mock-boosted animal which developed clinical disease. Instead this animal shows an immediate antibody response to the challenge in the weeks preceding the development of clinical MCF. These responses were observed in both neutralisation and ELISA assays (Figure 6.3).

The remaining calves (400821 and 500815) remained seropositive until the experiment termination with no clinical signs observed (212 days post inoculation). The general trend of antibody response was mirrored in both the ELISA and neutralisation assay. A decline in antibody response was seen in one animal following challenge with AlHV-1 (400821, Figure 6.4), while a rapid antibody response to the virus was seen in the second animal (500815, Figure 6.2). This suggests that the AlHV-1 vaccination did not offer significant protection against the subsequent development of clinical MCF, as both control and vaccinated groups showed cases of clinical disease following challenge with virulent virus. The ELISA offers a consistent demonstration of antibodies throughout the vaccination trial and therefore may be of benefit for routine testing of herds for the presence of antibody, whether clinical disease occurs or not. However, it cannot differentiate between acute, latent or recrudescent infections.

	400251		500815		200819		400821	
Day	**SNT**	**ELISA**	**SNT**	**ELISA**	**SNT**	**ELISA**	**SNT**	**ELISA**
0	0	0.013	0	0.012	0	0.029	0	0.023
7	360	0.054	512	0.08	3	0.030	3	0.071
14	512	0.036	720	0.038	45	0.070	11	0.09
21	360	0.107	360	0.218	45	0.149	128	0.186
28	720	0.478	1024	0.694	128	0.316	128	0.553
35	1024	0.778	1024	0.918	128	0.477	360	0.689
42	1024	1.027	360	0.978	360	0.653	360	0.854
49	1024	1.008	1024	1.185	180	0.583	512	0.881
56	1024	0.587	512	1.247	512	0.579	180	0.83
63	720	0.999	1024	1.154	720	0.581	1024	0.927
70	720	0.941	1024	1.315	512	0.784	360	1.01
77	1024	0.825	720	1.313	180	0.755	256	1.041
84	1024	1.011	360	1.303	512	0.701	180	0.88
91	**n/s**	**n/s**	**n/s**	**n/s**	**n/s**	**n/s**	**n/s**	**n/s**
98	1024	0.755	360	1.204	360	0.691	360	0.918
105	**n/s**	**n/s**	**n/s**	**n/s**	**n/s**	**n/s**	**n/s**	**n/s**
112	512	1.03	1024	1.191	256	0.605	90	0.813
119	512	1.002	1024	1.17	360	0.816	360	0.921
126	90	1.138	720	1.227	22	0.899	45	0.712
133	360	0.987	256	1.224	22	0.672	45	0.652
140	90	0.925	90	1.16	45	0.747	90	0.678
147	360	0.972	90	0.958	90	0.613	90	0.693
154	720	0.995	128	1.03	360	0.775	1024	0.824
168	512	0.715	1024	2.699	1024	1.427	360	0.725
175	ND	0.876	ND	2.504	ND	1.406	ND	0.592
182	ND	0.751	ND	2.422	-	-	ND	0.592
189	ND	1.375	ND	2.516	-	-	ND	0.669
198	-	-	ND	2.436	-	-	ND	0.59
205	-	-	ND	2.453	-	-	ND	0.722
212	-	-	ND	2.344	-	-	ND	0.626

Table 6.1: Analysis of experimentally AlHV-1 infected cattle by two diagnostics assays (Virus neutralisation assay and ELISA). Four animals, experimentally inoculated with MCFV were analysed by serum neutralisation test (SNT) and WC11-ELISA. n/s = days not sampled, ND = not tested. Vaccination occurred at day 0. Animal was boosted with AlHV-1 virus/chitosan on days 14 and 112, and challenged with virulent AlHV-1 (day 154). Table shows SNT titration results and ELISA OD values. The OD calculated represents the mean of the control antigen subtracted from the mean of the OD for positive antigen.

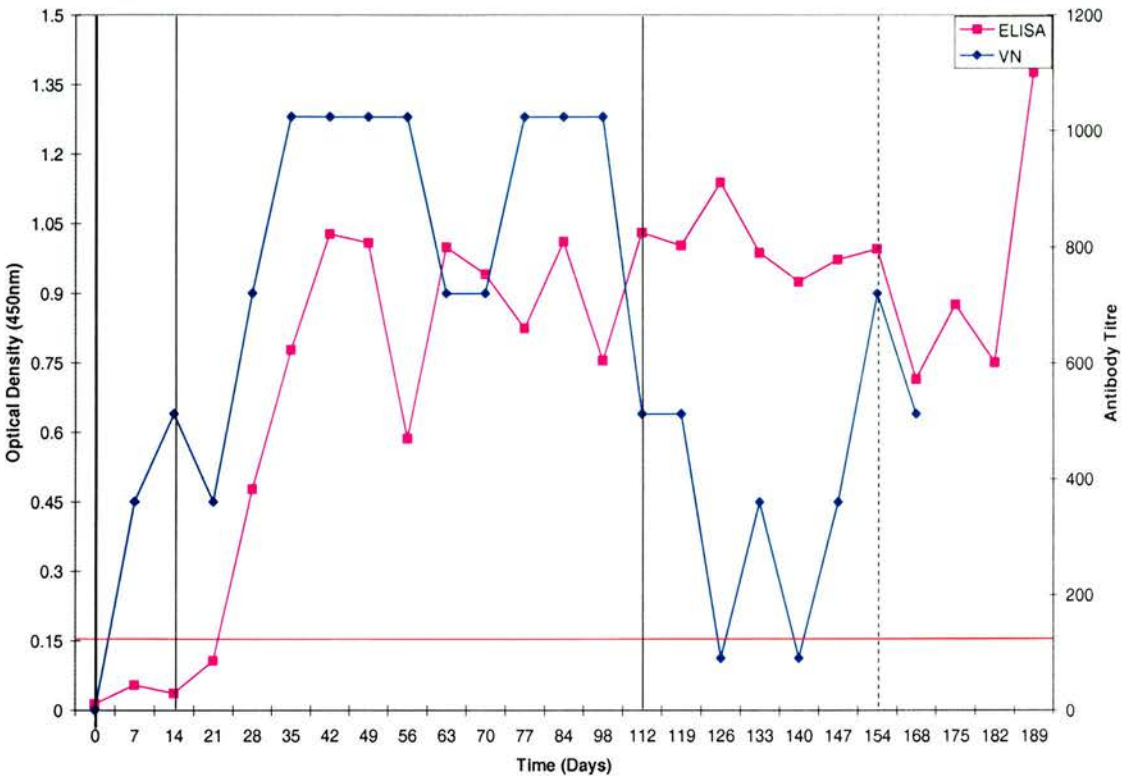


Figure 6.1. Immune response to AlHV-1.

Neutralisation assays (VN) and WC11-ELISA (ELISA), were used to measure MCF virus-specific antibody in plasma of calf 400251 following vaccination with attenuated AlHV-1 virus and challenge with virulent virus. Vaccination occurred at day 0. Solid black lines indicate time-points were the animal was boosted with AlHV-1 virus/chitosan (day 14, 112). Dotted line indicates time-point that the animal was challenged with virulent AlHV-1 (day 154), while the solid red line indicates WC11-ELISA cut-off value (>0.15 is considered positive). Animal died from MCF on day 43.

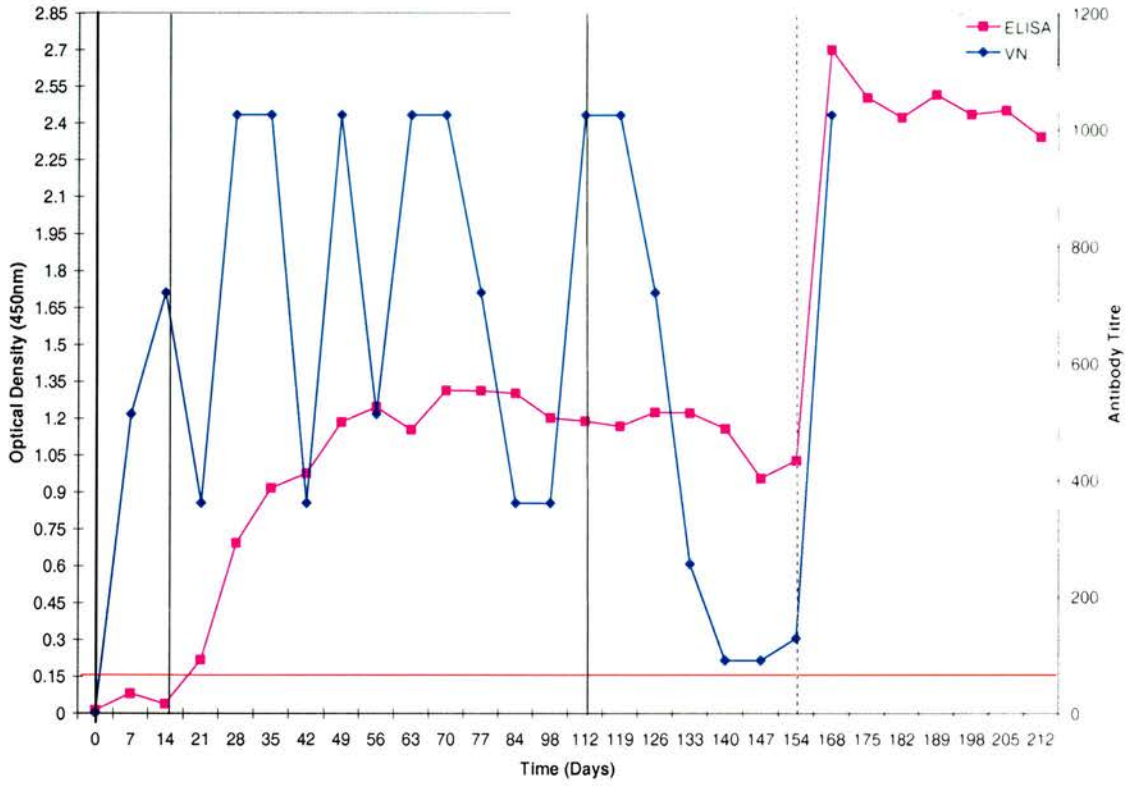


Figure 6.2. Immune response to AlHV-1.

Neutraisation assays (VN) and WC11-ELISA (ELISA), were used to measure MCF virus-specific antibody in plasma of calf 500815 following vaccination with attenuated AlHV-1 virus and challenge with virulent virus. Vaccination administered on day 0. Solid black lines indicate time-points were the animal was boosted with AlHV-1 virus/chitosan (day 14, 112). Dotted line indicates time-point that the animal was challenged with virulent AlHV-1 (day 154), while the solid red line indicates WC11-ELISA cut-off value (>0.15 is considered positive). Animal showed no clinical signs of MCF at end of experiment.

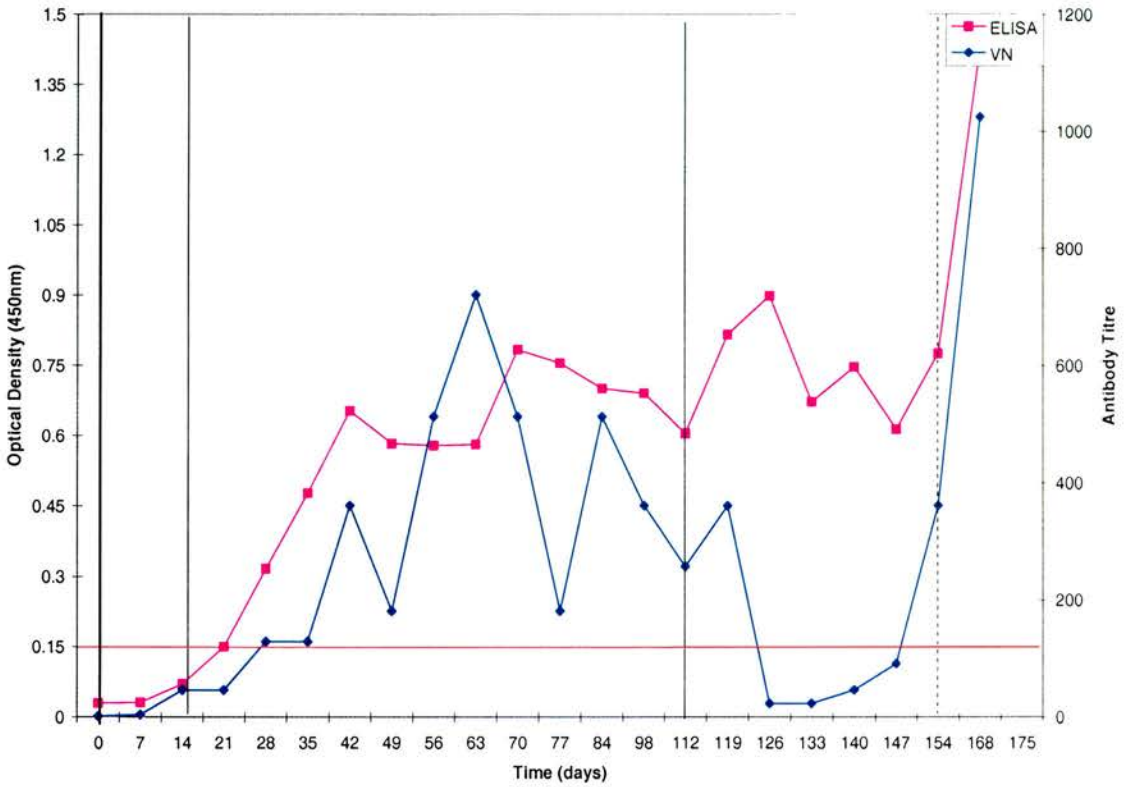


Figure 6.3. Immune response to AlHV-1.

Neutralisation assays (VN) and WC11-ELISA (ELISA), were used to measure MCF virus-specific antibody in plasma of calf 200819 following vaccination with attenuated AlHV-1 virus and challenge with virulent virus. Vaccine administered on day 0. Solid black lines indicate time-points were the animal was boosted with chitosan/medium (day 14, 112). Dotted line indicates time-point that the animal was challenged with virulent AlHV-1 (day 154), while the solid red line indicates WC11-ELISA cut-off value (>0.15 is considered positive). Animal died from MCF on day 23.

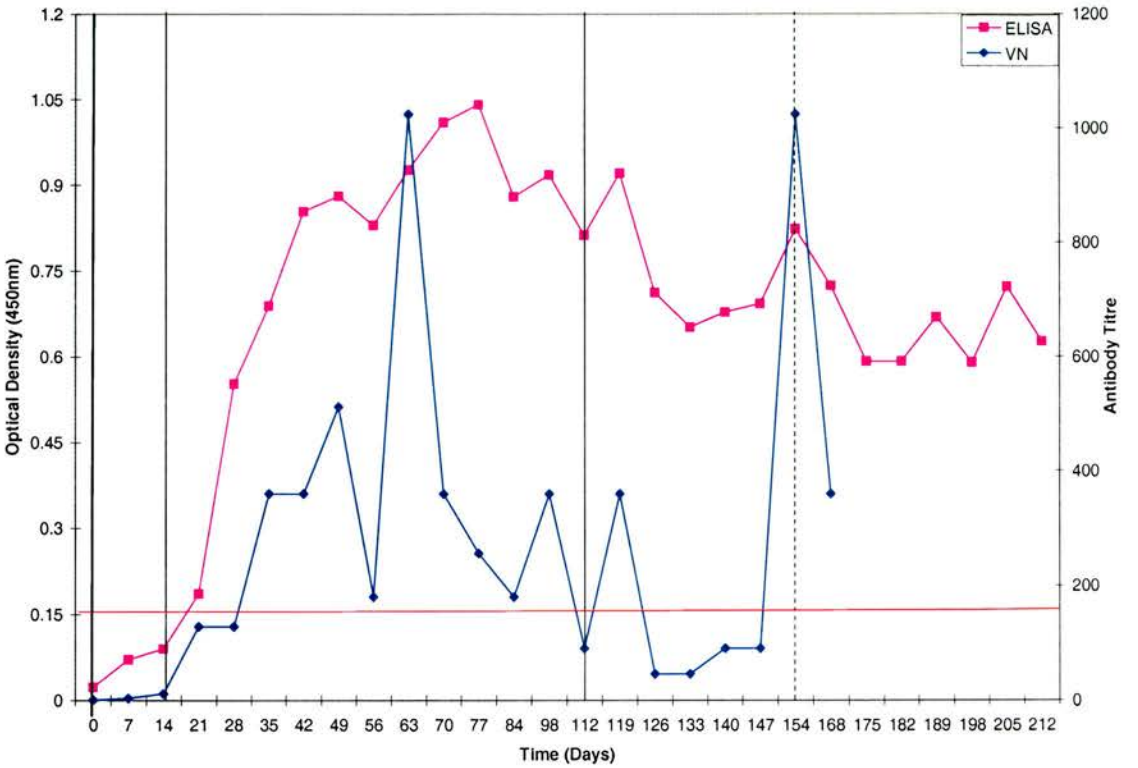


Figure 6.4. Immune response to AlHV-1.

Neutralisation assays (VN) and WC11-ELISA (ELISA), were used to measure MCF virus-specific antibody in plasma of calf 400821 following vaccination with attenuated AlHV-1 virus (day 0) and challenge with virulent virus. Solid black lines indicate time-points were the animal was boosted with chitosan/medium (day 14, 112). Dotted line indicates time-point that the animal was challenged with virulent AlHV-1 (day 154), while the solid red line indicates WC11-ELISA cut-off value (>0.15 is considered positive). Animal showed no clinical signs of MCF at end of experiment.

6.3.2. Validation studies

Clinical signs vary depending on the form of MCF contracted. Typically, clinical signs characteristic of the head and eye form include corneal opacity, pyrexia, mucopurulent nasal and ocular discharge and mucosal ulceration. The alimentary or intestinal form, being the second most recognised form of MCF, can be characterised by severe enteritis and diarrhoea. Definitive diagnosis remains reliant on histopathological examination, therefore, histopathology was used as the gold standard for the comparison of diagnostic tests, where applicable.

In 2006, cases of suspected MCF in domestic cattle were tested by four diagnostic tests; IFAT, CI-ELISA and WC11-ELISA serological tests and a real-time OvHV-2 PCR assay (Hussy *et al.*, 2001). As all samples were from suspected cases of MCF, all were submitted with clinical signs which were suggestive of MCF and therefore a confirmed diagnosis was required. In cases where histopathology was not performed, final veterinary diagnosis was sought from the submitting VI centre.

Parallel testing of forty-two serum samples by the four tests (Table 6.2) revealed between 57% and 76% concordance. Total concordance between the four tests was shown for 15 (36%) of the samples analysed. Of the forty-two samples selected, thirty-one (74%) were positive by PCR, nineteen (45%) were positive by IFAT, twenty-one (50%) were positive by CI-ELISA and nineteen (45%) were positive by WC11-ELISA. The agreement between the four assays exceeded 35%.

6.3.2.1. MCF-confirmed by Histopathological examination

Histopathological examination and clinical signs reported MCF in only seven samples. From the seven samples; all were positive by the OvHV-2 real-time PCR, one was positive by IFAT, three were positive by the CI-ELISA and three were positive by the WC11-ELISA (Table 6.3). Samples 16 and 18 showed agreement in three tests with discrepant results in the CI-ELISA (sample 16 (-22% inhibition) and IFAT (sample 18). Two samples (22 and 25) were positive by the CI-ELISA (26 and 34%) and PCR and negative by both the IFAT test and WC11-ELISA (0.04 and 0.01). These samples were submitted from a suspected MCF outbreak, where one animal in the herd underwent histopathological analysis. The examination reported sub-acute non- suppurative vasocentric meningoencephalitis, sub-acute hyperplastic lymphadenopathy, sub-acute non-suppurative cholangiohepatitis and acute multifocal abomasal ulceration. Based on these findings MCF was the confirmed diagnosis. A

Sample	DNA OvHV-2 PCR	Serology			Histopathology	Final Diagnosis
		IFAT	CI-ELISA	WC11-ELISA		
1	+	+	+	+	ND	MCF
2	+	+	+	+	ND	MCF
3	+	+	+	+	ND	MCF
4	+	+	+	+	ND	MCF
5	+	+	+	+	ND	MCF
6	+	+	+	+	ND	MCF
7	+	+	+	+	ND	MCF
8	+	+	+	+	ND	MCF
9	+	+	+	+	ND	MCF
10	+	+	+	-	ND	MCF
11	+	+	+	-	ND	MCF
12	+	+	+	-	ND	MCF
13	+	+	-	+	ND	MCF
14	+	+	-	+	ND	MCF
15	+	+	-	+	ND	MCF
16	+	+	-	+	+	MCF
17	+	-	+	+	ND	MCF
18	+	-	+	+	+	MCF
19	+	+	-	-	ND	MCF
20	+	+	-	-	ND	MCF
21	+	-	+	-	ND	MCF
22	+	-	+	-	+	MCF
23	+	-	+	-	ND	MCF
24	+	-	+	-	ND	MCF
25	+	-	+	-	+	MCF
26	+	-	-	+	+	MCF
27	-	+	-	+	ND	MCF
28	+	-	-	-	+	MCF
29	+	-	-	-	+	MCF
30	+	-	-	-	ND	MCF
31	+	-	-	-	ND	MCF
32	+	-	-	-	ND	MCF
33	-	-	+	-	ND	Unknown
34	-	-	+	-	ND	Unknown
35	-	-	-	+	ND	Unknown
36	-	-	-	+	ND	Unknown
37	-	-	-	-	ND	Unknown
38	-	-	-	-	ND	Unknown
39	-	-	-	-	ND	Unknown
40	-	-	-	-	ND	Unknown
41	-	-	-	-	ND	Unknown
42	-	-	-	-	ND	Unknown

Table 6.3: Detection of MCF infection in Field sera. Comparison of four diagnostic tests: PCR, IFAT, CI-ELISA and WC11-ELISA. + indicates MCF, - MCF is undetected and ND means analysis not done.

further two samples from this outbreak were only positive by the OvHV-2 real-time PCR, with all negative serological assay results (sample 28 - 0.03, -7% and sample 29- 0.02, 5%). The remaining sample, 26, showed agreement between WC11-ELISA (0.16 AU) and PCR, with negative results by both the IFAT test and CI-ELISA (3%). This sample was confirmed as an alimentary/intestinal form of MCF.

Sample Number	PCR	Serology		
		IFAT	CI-ELISA	WC-ELISA
16	+	+	-	+
18	+	-	+	+
22	+	-	+	-
25	+	-	+	-
26	+	-	-	+
28	+	-	-	-
29	+	-	-	-

Table 6.3: Histopathological examination confirming MCF (n = 7). Comparison of four diagnostic tests to histopathological examination: PCR, IFAT, CI-ELISA and WC11-ELISA. + indicates MCF, - MCF is undetected.

6.3.2.2. Comparison of DNA and serological assays

In the group of 42 samples, a positive signal was detected by PCR in a total of thirty-one cases, including all seven positive by histopathology and twenty-four positive by final veterinary diagnosis. No signal was detected for ten cases, in which the final diagnosis eliminated MCF (Table 6.4). Interestingly no signal was detected for one further case (Number 27), which was positive by two serological tests (IFAT and WC11-ELISA).

This study revealed that the OvHV-2 PCR assay showed a good correlation with the histopathological evidence and final veterinary diagnosis, and therefore serological test results will be compared to the PCR assay results for all samples. It should be recognised, however, that cases of MCF and animals recovered from MCF have been shown to be serologically positive but PCR negative. Therefore PCR is not a perfect "gold standard" for this analysis. Results from the three serological assays were pooled and samples were classed as positive where a positive result was found in one or more serological test, and were compared to the OvHV-2 PCR results (Table 6.4). Twenty-six samples were considered positive by both DNA and serology. Serology test sensitivity was determined as 77% as a result of five serology-negative/ DNA-positive samples results. On examining these five animals

	Final Veterinary Diagnosis			
	Positive	**Negative**	**Total**	**% Agreement**
PCR Result				
Positive	31[a]	0[b]	31	97.6
Negative	1[c]	10[d]	11	
Total	32	10	42	

Table 6.4: Comparison of the relative sensitivity and specificity of the OvHV-2 real-time PCR assay compared to a final veterinary diagnosis. Test sensitivity of 97% (a/a+c), with a specificity of 100% (d/d+b).

	DNA			
	Positive	**Negative**	**Total**	**% Agreement**
Serology Results				
Positive	26[a]	5[b]	31	76.2
Negative	5[c]	6[d]	11	
Total	31	11	42	

Table 6.5: Comparison of the relative sensitivity and specificity of the serological assays compared to the OvHV-2 real-time PCR assay. The serological assays gave a relative sensitivity of 77% (a/a+c) and specificity of 84% (d/d+b).

to final veterinary diagnosis, four were submitted from a suspected MCF outbreak where animals in the herd underwent histopathological analysis and confirmed MCF. A possible explanation for the negative-serology result may be because these cattle, with possible clinical signs, were tested prior to an antibody response being established. Low antibody levels might therefore not be detected in the blood sample. Serology showed 84% specificity when compared to DNA results, with five samples serology-positive/DNA-negative. Of these five samples, four animals were positive in only one serological test and are assumed false-positive. Final veterinary diagnosis for these animals was not conclusive. The remaining six samples were negative in all DNA and serology assay tests.

Next, the efficiency of each serological assay, for the diagnosis of MCF, was tested independently by determining their relative sensitivity and specificity, using the OvHV-2 PCR as the "gold standard".

6.3.2.3. Comparison of IFAT with OvHV-2 PCR for detection of MCFV

By IFAT, antibodies against MCFV were detected in nineteen serum samples. One sample reacted positively in the IFAT but appeared negative by PCR, while a further thirteen samples reacted negatively by IFAT but appeared positive by PCR. The remaining ten samples gave negative results in both assays. Employing PCR as the "Gold standard", IFAT gave a sensitivity of 95% and a specificity of 43% and showed an overall agreement of over 66% (fair agreement, k = 0.36) (Table 6.6). The fourteen discrepant samples were compared to final veterinary diagnosis. Of the thirteen samples OvHV-2 real-time PCR positive/ IFAT-negative, six animals were confirmed MCF by histopathology. A further seven animals were suspected MCF cases based on final veterinary diagnosis. Of these seven animals, one showed classical symptoms for the head and eye form of MCF. A second animal showed classical symptoms for intestinal MCF. The remaining animals exhibited characteristic MCF symptoms which included pyrexia, purulent nasal/ocular discharge and lymphopaenia.

The OvHV-2 real-time PCR negative/ IFAT-positive sample had MCF confirmed by histopathology with clinical signs of ulcerative stomatitis, pyrexia and enteritis. This sample was additionally a low WC11-ELISA positive (0.163)/CI-ELISA negative (-81%).

	IFAT			
	Positive	**Negative**	**Total**	**% Agreement**
PCR Result				
Positive	18[a]	13[b]	31	66.6
Negative	1[c]	10[d]	11	
Total	19	23	42	

Table 6.6: Comparison of the relative sensitivity and specificity of IFAT compared to the OvHV-2 real-time PCR assay. IFAT gave sensitivity of 95% (a/a+c) and specificity of 43% (d/d+b).

6.3.2.4. Comparison of CI-ELISA with OvHV-2 PCR for detection of MCFV

Nineteen samples reacted positively by both the OvHV-2 real-time PCR assay and the CI-ELISA. Twelve samples were PCR-positive/ CI-ELISA-negative while a further two samples reacted PCR-negative/ CI-ELISA-positive. The remaining nine samples gave negative results by both assays. Employing PCR as the

"Gold standard", the CI-ELISA gave a sensitivity of 61% and a specificity of 82% and showed an overall agreement of over 66% (fair agreement, k = 0.34) (Table 6.7). Discrepant samples were compared to a final veterinary diagnosis. Of the twelve samples positive by PCR but negative by CI-ELISA, four were MCF confirmed by histopathology, while the remaining eight samples were suspected MCF cases based on final veterinary diagnosis. Based on clinical evidence, the two samples positive by CI-ELISA (29 and 33%) but negative by PCR, were likely MCF-negative (IFAT negative/WC11-ELISA negative). Signs included pyrexia and ocular discharge.

	PCR			
	Positive	**Negative**	**Total**	**% Agreement**
CI-ELISA Result				
Positive (>25%)	19[a]	2[b]	20	66.6
Negative (<25%)	12[c]	9[d]	22	
Total	31	11	42	

Table 6.7: Comparison of the relative sensitivity and specificity of the CI-ELISA compared to PCR. CI-ELISA sensitivity of 61% (a/a+c) and specificity of 82% (d/d+b)

6.3.2.5. Comparison of WC11-ELISA with OvHV-2 PCR for detection of MCFV

Sixteen samples reacted positively by both the OvHV-2 RT PCR assay and the WC11-ELISA. Three samples reacted positively in the WC11-ELISA, but reacted negatively by PCR. A further fifteen samples were WC11-ELISA negative/ PCR-positive. The remaining eight samples gave negative results by both assays (Table 6.8). Of the fifteen samples PCR-positive/ WC11-ELISA negative, five samples were negative by both IFAT and WC11-ELISA while two samples were negative by both ELISA assays. Three samples were only negative by the WC11-ELISA. All of these discrepant false-negative samples were confirmed MCF by either histopathology (4 samples) or final veterinary diagnosis (11 samples). The remaining five samples were negative in all serological assays. Employing PCR as the "Gold standard", the WC11-ELISA gave a sensitivity of 52% and a specificity of 73% and showed an overall agreement of over 57% (fair agreement, k = 0.22) (Table 6.8).

Three samples were identified as PCR-negative/ WC11-ELISA positive, one (sample 27) of which was also positive by IFAT. This sample was positive by

	PCR			
	Positive	**Negative**	**Total**	**% Agreement**
ELISA Result				
Positive (>0.15)	16[a]	3[b]	19	57
Negative (<0.15)	15[c]	8[d]	23	
Total	31	11	42	

Table 6.8: Comparison of the relative sensitivity and specificity of the WC11-ELISA compared to PCR. WC11-ELISA gave a sensitivity 52% (a/a+c) and specificity of 73% (d/d+b).

nested-PCR (Baxter *et al.*, 1993). An explanation for this real-time PCR-negative result could be that the animal had a low viral load, below the threshold of detection by the real-time PCR. This would suggest that this assay is not as sensitive as the nested PCR. All other OvHV-2 real time PCR negative samples where tested by nested PCR and were confirmed as MCF-negative. Of the two remaining WC11-ELISA positive/ PCR negative samples, one was diagnosed MCF negative. The second animal remained undiagnosed, although it was assumed to be MCF negative. Both samples were negative by IFAT and CI-ELISA.

6.3.3. Cross-reactivity

Potential cross-reactivity of the serological MCF tests with bovine herpesvirus-4 was also analysed using a direct BoHV-4 ELISA (Bio-X Diagnostics). Of the 42 sera tested, only two samples were BoHV-4 positive. Of these samples, one was positive by the CI-ELISA (29%), but negative by WC11-ELISA, IFAT and PCR. The second sample was positive by both MCF ELISAs (low WC11-ELISA positive 0.17, CI-ELISA positive 31%) and PCR. This result confirms previous results (section 5.2.2.4) that BoHV-4 and MCF-positive detection are not linked but may occur coincidentally.

6.4. Discussion

The initial objective of this study was to follow a longitudinal study of cattle experimentally immunised with AlHV-1. The infection status of each animal was examined from pre-inoculation to termination, using two diagnostic methods: virus neutralisation assays and the WC11-ELISA. The virus neutralisation assays showed high and persistent levels of virus-neutralising antibodies were demonstrable, especially in calves intranasally boosted with AlHV-1. The WC11-ELISA showed corresponding patterns of antibody response for each animal. The main difference between the tests was the sensitivity, where the neutralisation assay detected specific antibody at least one week earlier than the WC11-ELISA. This lack of sensitivity may be explained by the use of diluted test serum (1/100) in the ELISA, where serum is titrated from neat in the neutralisation test. By changing the serum dilution in the ELISA or possibly by using a similar titration method, a better indication of the ELISA sensitivity compared to the neutralisation assay might be obtained.

In this longitudinal study the ELISA demonstrated an antibody response in all animals throughout the vaccination trial and therefore may be of benefit for epidemiological studies in routine testing herds for the presence of antibody, whether clinical disease occurs or not. An obvious limitation of the ELISA in this context was its inability to differentiate between acute, latent and recrudescent infections or between neutralising and non-neutralising antibody. The ELISA antigen was derived from lytic BT infection. Of further consideration was the use of experimental animals. As the animals were vaccinated their immune response may not represent a natural infection.

As a further validation measure, parallel DNA and serum samples submitted for MCF testing were collected and analysed to compare the diagnostic methods available for the detection of MCF, namely an OvHV-2 specific diagnostic PCR, IFAT, CI-ELISA and WC11-ELISA. IFAT and the WC11-ELISA employ a crude lysate antigen for the detection of antibodies against MCF viruses. This enables the test to detect antibodies specific for a wide range of structural components of the virus. The CI-ELISA is based on a monoclonal antibody (MAb 15-A) specific to a single epitope conserved among MCFV isolates (Li *et al.*, 1994, 2005). Histopathological examinations revealed that the 7 tested cattle displayed lesions typical of MCF. In cases where all four diagnostic tests were in agreement (n = 15), no histopathological data was available, while in sixteen cases MCF was diagnosed based on clinical evidence. The remaining four cases were diagnosed MCF negative

based on a final diagnosis supplied by the veterinarian. Results indicate that the WC11-ELISA is a useful method for the determination of antibodies to AlHV-1 in cattle, and showed good agreement with established diagnostic methods for detecting MCF. Of the MCF-infected animals diagnosed by either histopathology or clinical evidence, 97% also had positive test results by PCR, 59% had positive test results by IFAT, 61% had positive test results by CI-ELISA and 53% also had positive test results by WC11-ELISA. Of the animals clearly negative by either histopathology or clinical evidence, 100% also had negative test results by PCR, 100% had negative test results by IFAT, 80% had negative test results by CI-ELISA and 80% also had negative test results by WC11-ELISA (Table 6.9).

The OvHV-2 PCR showed over 97% concordance with histopathology and final diagnosis and therefore appeared to be the most reliable diagnostic test analysed. One explanation for the PCR showing enhanced diagnosis when compared to the serological tests is that animals, particularly those involved in an outbreak, may have acute infections with virus being present in the blood but the immune response has not developed a sufficient antibody response to be detected in serum. Certainly, in AlHV-1 challenged animals, virus is detected in the blood before clinical signs are seen (G. Russell, Unpublished finding).

Test	Test Sensitivity (%)	Test Specificity (%)	% Agreement
PCR	97	100	97
IFAT	59	100	59
CI-ELISA	59	80	61
WC11-ELISA	55	80	53

Table 6.9: Comparison of test sensitivity and specificity for all diagnostic tests, compared to a final diagnosis.

Antibody to OvHV-2 has only been detected by using AlHV-1 as the source of antigen. Antibody to AlHV-1 can be detected in 70–80% of clinically affected cattle by IFAT procedures, but generally is not present in affected deer or animals that develop acute or peracute disease and succumb within a few days of the onset of symptoms (Klieforth *et al.*, 2002). A report by Herring *et al.*, (1989) investigating virus neutralisation by serum from animals with terminal MCF, suggested antibody was either absent or present only at a low titre. Following AlHV-1 infection, the humoral response of the bovine host affected with acute MCF is ineffective in preventing disease (Herring *et al.*, 1989). This could suggest cattle only react to a

limited number of viral components, either as a result of low levels of viral replication or an incomplete expression of the full genetic range of AlHV-1 antigens. It could also indicate that the immune response is severely compromised by virus infection.

Serological tests such as the CI-ELISA may indicate previous infection but may not necessarily indicate current infection. It can also not discriminate between acute infection and long-term infections such as might be found in chronic or recovered cases of MCF (Powers *et al.*, 2005).

Examining the serological assays, the number of seropositive cattle detected by IFAT was greater than the number detected by either of the ELISA assays, showing 95% sensitivity but only 43% specificity when compared to the PCR assay. This finding reinforces the supposition in chapter six that the number of seropositive cattle detected by IFAT was usually equal to or greater than the number detected by the two ELISA tests (section 6.2.4).

In contrast with the findings in the establishment of the WC11-ELISA (chapter five), only slight positive agreement between the magnitude of reaction in the CI-ELISA and the WC11-ELISA has been demonstrated, with both tests detecting animals with MCF with a degree of accuracy and agreement. Parallel testing of forty-two field serum samples from cattle, revealed over 57% concordance between CI-ELISA and WC11-ELISA. In contrast to the 97.3% concordance shown in chapter five, the number of seropositive cattle detected by the CI-ELISA was greater than the number detected by the WC11-ELISA, showing 61% and 52% sensitivity and 73% and 82% specificity respectively.

This result implies that the WC11-ELISA was not as accurate in the detection of MCF as the CI-ELISA in this group of samples. Of the ten samples that were CI-ELISA positive/ WC11-ELISA negative, two cases were confirmed by histopathology and six were confirmed by a final veterinary diagnosis. The remaining two samples may be considered false-positive as a final diagnosis of MCF was not reached. Of the eight samples that were CI-ELISA negative/ WC11-ELISA positive, two cases were confirmed by histopathology and four were confirmed by a final veterinary diagnosis. Similar to the CI-ELISA, two samples may be considered false-positive as a final diagnosis of MCF was not reached, and no other test showed virus or antibody present. Therefore the fact that there were 33% discrepant samples between the tests was not simply due to the accuracy of one test in particular. Both the CI-ELISA and WC11-ELISA incorrectly diagnosed 13-15 positive samples (31%

and 36% respectively). Furthermore, thirteen samples were also incorrectly diagnosed by IFAT (31%). It is possible that these cases represent animals previously exposed to MCF viruses, with no or low levels of detectable antibody to MCF.

Although the PCR assay proved the most reliable test in this study, samples where an antibody response was detected but no virus DNA was found were observed (five samples PCR negative/serology positive). Although in four of the five samples, the positive serology result appeared to be false-positive results, one sample was confirmed positive by histopathology or final veterinary diagnosis. A possible explanation for the negative PCR result may be due to the sensitivity of the real-time PCR assay, as this sample was confirmed positive by the more sensitive nested PCR.

It was generally observed that in clinical cases of MCF, viral DNA was readily detected in the blood and tissues of the infected animal (Baxter *et al.*, 1993; Li *et al.*, 1995; Collins *et al.*, 2000). Some validation studies, however, have shown contrasting results where the PCR result differs from serology or histopathology. Powers *et al.*, (2005) showed a different observation in a study of cattle from a farm with history of MCF, where serology-positive/DNA-negative animals were present. This may indicate animals which have had past infections and therefore may have an antibody response, but no viral DNA. Other possible explanations may include the viral DNA being present at a low level, undetectable by PCR. This could represent a form of latent infection of an animal, and therefore levels of virus circulating in the blood would be considerably lower. It is also suggested asymptomatic infections may not cause the production of virus and therefore indicates the PCR assay may fail in some cases to consistently detect OvHV-2 infection (Powers *et al.*, 2005).

Additionally, samples where no antibody response was detected, but viral DNA was determined by PCR, were also found (five samples PCR positive /serology negative). In these five cases, all three serology tests gave a negative result. Lahijani *et al.*, (1994) studied the application of a PCR test in exotic ruminants and showed samples which were positive by DNA but negative by serology. Samples tested by a virus neutralisation assay and PCR showed that some animals had no detectable neutralising antibodies present in the blood. It was suggested that the animals were either sampled during a non-replicative stage in the virus life cycle or that immunological tolerance had been established. Li *et al.*, (1995) suggested that a further explanation for serology negative animals may be the lack of sufficient time to mount an immune response due to the rapid clinical progression of infection. With particular reference to the CI-ELISA, it is possible that individual animals may lack

humoral immune response to the viral antigen targeted by the monoclonal antibody used in the assay (Li *et al.*, 1996), as appeared to be the case for two samples in chapter five.

Further validation studies have shown concordance between the four diagnostic methods tested. Comparative studies demonstrated that IFAT, CI-ELISA and WC11-ELISA were specific for MCFV antibody, although in this study PCR appeared to be more reliable for diagnosis of clinical MCF. Therefore these results agree with previous studies comparing serological assays to histopathology examination and PCR (Li *et al.*, 1996; Muller-Doblies *et al.*, 1998; Powers *et al.*, 2005). Few samples were serologically positive/PCR negative, while the majority were PCR positive/serology negative. This finding supports the view that animals may be infected and sampled before a serological response has developed, or the animal develops MCF before the serological response is able to occur. A much lower number of chronic, latent or recovered cases of MCF occur, where the viral load in blood has reduced below the level of detection by Real-time PCR.

The data obtained in this comparative study indicate the WC11-ELISA as a useful, specific and economical serological assay for the detection of antibodies against MCFV. This study also demonstrated that the WC11-ELISA is capable of detecting the presence of anti-MCF antibodies in cattle prior to the appearance of clinical MCF, so that sources of infection may be more readily established.

Chapter Seven

Antigen Identification

7.1. Introduction

Pathogenesis and epidemiology of OvHV-2 is still not entirely understood, fundamentally due to the lack of effective detection methods for the etiologic agent or antibody against it. Furthermore, serological cross-reactivity remains a concern. Early work to determine AlHV-1 proteins with diagnostic significance demonstrated a preliminary characterisation of virus-induced proteins using serum from infected and uninfected rabbits (Adams and Hutt-Fletcher, 1990). Virus-induced proteins from the AlHV-1 virus strain WC11 were shown at 48, 78, 95, 105, 110, 115, 130 and 145kDa. These proteins were not detected in uninfected animals. A more definitive characterisation of AlHV-1 virus-induced proteins was then performed with monoclonal sera. Fifteen monoclonal antibodies were obtained and immunoprecipitation studies grouped the antibodies into three types (Adams and Hutt-Fletcher, 1990). Type 1 and 2 antibodies precipitated proteins of 45 and 145kDa, while the type 3 antibodies immunoprecipitated a protein complex (gp115 complex), which contained five polypeptides (48, 78, 105, 110 and 115kDa) as shown by SDS-PAGE run under reducing conditions.

As a basis for a specific serological assay, an attempt was made to identify an epitope conserved among all isolates of MCF viruses, by using a monoclonal antibody (MAb) produced against a previously reported isolate of MCF virus recognizing the gp115 complex (Li *et al.*, 1994). The identified MAb (15-A) bound to a conserved epitope on four isolates of MCF virus. On analysis by immunoprecipitation, MAb 15-A was located on the viral glycoprotein complex (gp115), which contains polypeptides of 45, 78, 105, 110 and 115kDa. Further analysis of the components of MCFV was required for the purpose of developing a diagnostic test (Li *et al.*, 1995). Lysates from AlHV-1 infected cells were immunoprecipitated with polyclonal antisera from either an AlHV-1 naturally infected wildebeest or from AlHV-1 experimentally-infected sheep. When analysed by western blot, eleven major bands were identified, and constituted six major antigenic groups, one of which was the gp115 complex (115, 110, 105, 78 and 48kDa). Viral proteins at molecular masses of 17, 24, 34, 36, 110 and 130kDa were additionally identified (Li *et al.*, 1995). Potential candidates, which may be employed in diagnostic tests were determined from this study. The earlier work by Li *et al.*, (1994) identified an epitope conserved among isolates of MCFV and defined by the MAb 15-A which suggested its usefulness in a diagnostic assay. MAb 15-A binds to an epitope on the glycosylation-dependent glycoprotein complex gp115. Analysis of

MAb 15-A showed the antibody was MCF-positive by IFAT and immunoprecipitation and was inhibited by sera. Furthermore, analysis of the gp115 complex under reducing conditions showed that protein fragments at 115, 110 and 105 were related by glycosylation. Under reducing electrophoretic conditions, the gp115 complex reduced into bands of 48 and 78kDa in size. The proteins identified in these studies (Li *et al.*, 1994; Li *et al.*, 1995) were consistent with previous studies of AlHV-1 envelope proteins (Adams and Hutt-Fletcher, 1990).

Analysis using the competitive ELISA approach with field sera suggested cattle naturally infected with OvHV-2 produce antibodies to AlHV-1 that can compete with monoclonal antibodies, in binding to a virus-infected cell lysate (Adams & Fletcher, 1990; unpublished findings; Li *et al.*, 1994). This suggests that the gp115 complex is highly conserved and may be considered important to study in order to gain a further insight into the understanding of AlHV-1 pathogenesis (Adams & Fletcher, 1990).

The sequencing of both the AlHV-1 (Ensser *et al.*, 1997) and the OvHV-2 genomes (Hart *et al.*, 2007) provides an opportunity to identify genes unique to the MCF viruses and particularly potential diagnostic antigens. As an ELISA (Fraser *et al.*, 2006), based on a crude AlHV-1-infected cell lysate, was developed as part of this study (described in chapter five), it would be of particular interest to identify the antigens recognised by sera from clinically infected MCF-positive animals. To develop a specific diagnostic assay to detect MCF in infected animals, the approach of identifying viral components specific to AlHV-1 may prove valuable. Specific antibody or antigen can be separated from antisera and crude extracts by methods such as affinity purification, or conventional chromatography combined with western blotting.

This chapter describes three approaches used to identify potential MCFV antigens. In the first approach, AlHV-1 crude antigens were immobilised on Sepharose and used to isolate antibodies specific for those antigens in order to identify and purify AlHV-1 antigens with diagnostic significance. The second approach involved the use of IgG fractions isolated from MCF positive sera and immobilised on Sepharose to bind specific AlHV-1 antigens. The final approach investigated the use of a variety of conventional chromatographic techniques to isolate MCF specific antigens from cells infected with AlHV-1.

7.2. Materials and methods

7.2.1. Preparation of whole cell lysate antigens

Crude whole virus antigen was prepared from bovine turbinate (BT) cells grown in 150cm^2 flasks using Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% foetal bovine serum, 1% Glutamine and 1% penicillin and streptomycin. Cell monolayers were inoculated, at a multiplicity of infection (MOI) of 0.1 PFU/cell, with 4ml of the cytopathic isolate of MCF causative agent, AlHV1 strain WC11 (Plowright *et al.*, 1965). This was incubated for one hour before the addition of 100ml of medium with 2% FBS. When 90-100% of the cells showed virally-induced cytopathic effect (rounding of infected cells), supernatant was removed and cells were harvested into phosphate buffered saline (PBS). Cells were sedimented by centrifugation at 2000 x g for 10 minutes (4°C) and resuspended in 0.5ml of SDS-PAGE sample buffer. The cell lysate was then heated at ~85°C for 5 minutes, centrifuged for 10 minutes at 1000 x g, before being analysed by gel electrophoresis and western blot. Whole cell lysates from BT cells infected with herpesviruses; BHV-1, BHV-2 and BoHV-4; were prepared in a similar manner. Control antigen was prepared from mock-infected cells. Cells in the control flasks were detached by gently scraping the flask. The supernatant was then harvested and centrifuged at 2000 x g for 10 minutes (4°C). The cell lysate was then resuspended in 0.5ml of SDS-PAGE sample buffer.

Crude whole virus antigen was prepared from the BJ1035 LGL cell-line from an OvHV-2-infected animal (Shock *et al.*, 1998). BJ1035 was grown and maintained as described in section 2.3.1. Cells were resuspended in the flasks by pipetting, and the supernatant harvested. Cells were sedimented by centrifugation at 2000 x g for 10 minutes (4°C) and resuspended in 0.5ml of SDS-PAGE sample buffer. The cell lysate was then heated at ~85°C for 5 minutes, centrifuged for 10 minutes at 1000 x g, before being analysed by gel electrophoresis and western blot.

7.2.1.1. Preparation of whole cell lysate antigens

Crude whole virus antigen was prepared from bovine turbinate (BT) cells grown in 150cm^2 flasks using Iscove's Modified Dulbecco's Medium (IMDM) supplemented as stated in section 7.2.1. Cell monolayers were inoculated, at a multiplicity of infection (MOI) of 0.1 PFU/cell, with 4ml of the cytopathic isolate of MCF causative agent, AlHV1 strain WC11 (Plowright *et al.*, 1965). When 90-100% of the cells showed virally-induced cytopathic effect (rounding of infected cells),

supernatant was removed and cells were harvested into phosphate buffered saline (PBS). Cells were sedimented by centrifugation at 2000 x g for 10 minutes (4°C) and resuspended in PBS. Control antigen was prepared using the same protocol, using mock-infected BT cells.

7.2.2. Western Blotting

Gel electrophoresis and western blotting were performed (as described in sections 2.5.2. and 2.5.6) to determine the predominant proteins, present in the infected cell lysate, and recognise sera from MCFV infected cells. The western blots were performed as described in Chapter 2 (section 2.5.5). After transfer, non-specific binding sites on 5mm-wide nitrocellulose strips were blocked using 3% non-fat milk in high-salt wash buffer for 30 minutes at room temperature (22±5°C). The nitrocellulose strips were then incubated for a further 60 minutes at room temperature with a 1/100 dilution of test serum. Strips were washed to remove excess test antibody (3× 10 minutes in wash buffer) followed by incubation with HRP-labeled rabbit anti-bovine IgG for 60 minutes at room temperature (1/1000 dilution). Chemiluminescent substrate (Pierce SuperSignal West Dura) was applied to the membrane prior to exposure to x-ray film. The exposed film was developed using an automatic film processor (Compact X2: Xograph Imaging systems, Wiltshire, UK).

7.2.3. Coupling to HiTrap Column

Affinity chromatography, utilising an immobilised AlHV-1 antigen lysate, was used enrich antigen-specific antibodies from anti-MCF serum. Antigens were covalently bound to a column, and antibodies, specific to these bound antigens, were bound and isolated. HiTrap columns are designed for the covalent coupling of ligands containing primary amino groups, based on cross-linked agarose bead-resin (6% sepharose, 34μm mean particle size).

Briefly, crude antigen lysate, prepared as described in section 7.2.1.1, was dialysed against coupling buffer (0.1M $NaHPO_4$, 0.5M NaCl, pH 8.3). The dialysed antigen (1.2μg) was covalently coupled to an NHS activated Sepharose 1ml HiTrap column (GE Healthcare, Munich, Germany) following the manufacturer's instructions. The column was washed for several cycles with 3× 2mls 0.5M ethanolamine, 0.5M NaCl, pH8.3, followed by 3× 2mls 0.1M sodium acetate, 0.5M NaCl, pH4. The efficiency of coupling was checked by following the manufacturer's calculation (GE Healthcare: Instructions 71-7006-00 AT);

Coupling solution loaded (A_{280} × ml) = A

(Coupling solution × volume of coupling solution loaded).

Amount not coupled (A_{280} × ml) = B

(Post-coupling wash × volume of post-column wash × 2).

Percentage coupling yield: (A–B)/A × 100.

The column (1ml) was then equilibrated with coupling buffer (0.1M $NaHPO_4$, 0.5M NaCl, pH 8.3) and incubated for 30 minutes at room temperature. The antigen-HiTrap column was attached to an Akta FPLC system and 50mls (1:10 dilution) of crude serum was passed through the column. Bound antibodies were then eluted with 0.1M glycine (pH 2.8), neutralized with 1M Tris (pH9) and analysed by gel electrophoresis and western blotting. Samples were stored at -70°C.

Affinity chromatography was also performed using an immobilized MCF-positive purified IgG to enrich specific antibody-antigen binding.

7.2.4. Purification of MCFV specific immunoglobulin G (IgG) from serum

In order to minimise non specific binding, IgG was purified from serum. Serum samples (0.5ml), diluted 10 fold in 50mM Tris, 150mM NaCl pH 8.3 (TBS) and clarified by filtration through a 0.45μM membrane filter (Sartorius, Germany), were applied to a 1ml Protein-A HiTrap column (GE Healthcare) equilibrated with TBS. Bound IgG was eluted with 0.1M glycine pH 2.8 into neutralising 1M Tris pH 8.5. Prior to analysis, purified IgG was stored at -70°C.

7.2.5. Gel filtration - Superdex200

AlHV-1 WC11 crude lysate (0.5ml 1.2ng/ml) was applied to the Superdex200 column on the Akta FPLC system (described in section 2.7.2). The column was equilibrated with 0.04% CHAPS in PBS (pH8.2) to minimise protein aggregation. Fractions (1ml) were collected and fractions corresponding to protein peaks, as measured by absorbance at 280nm, were analysed by gel electrophoresis and western blotting, using an MCF-positive serum sample as a probe. Samples were stored at -70°C.

7.2.6. Ion exchange – Q-Sepharose

Ion exchange chromatography separates molecules based on differences in pI electrostatic potential. Elution was achieved by increasing the ionic strength of the column buffer by applying a linear salt gradient from 20mM Tris (pH 8.2) to 20mM Tris 1M Nacl (pH 8.2).Gel filtration samples that were reactive with MCF serum were further separated by Q-Sepharose. Prior to application to the column, samples were dialysed against 20mM Tris (pH8.2). The protein mixture was separated by increasing the NaCl concentration of the buffer and fractions (1ml) were collected. Absorbance peak fractions were analysed by SDS-PAGE and western blotting.

7.2.7. WC11 virus extraction using alternative detergents

WC11 supernatant from a 150cm^2 flask was removed and virus pelleted in an ultracentrifuge at 75000 x g for 16 hours. The supernatant was discarded and the virus and insoluble cellular material resuspended in 100µl of buffer containing a detergent (either: 1% Triton X-100, 1% NP40, 0.8% CHAPS/TBS or in TNE buffer plus 1.5M NaCl or in 1× sample loading buffer). The solubilised pellets were then incubated for 1 hour on ice and centrifuged at 13000 x g for 15 minutes. The supernatant was removed, and aliquots were stored at -20°C. The remaining cell pellet was resuspended in 20µl of 1× sample loading buffer.

The samples from different extraction methods were run on 10% tris-glycine polyacrylamide gels under reducing conditions, and were analysed by chemiluminescent western blot using MCF-positive sera. As the 1.5M NaCl-extracted lysate had a salt concentration too high to be run on a polyacrylamide gel, 10% (w/v) Trichloroacetic acid (TCA) was used to precipitate the protein from the salt solution. 1ml of 10% ice-cold TCA was added to 50µl of supernatant and incubated for 30 minutes on ice. Samples were centrifuged at 13000rpm for 10 minutes. The supernatant was then removed and the pellet resuspended in 1× sample loading buffer.

This work was done in collaboration with David Deane and Ann Percival, MCF group, Moredun Research Institute.

7.3. Results

7.3.1. Identification of MCFV antigens by western blot.

7.3.1.1. Analysis of bovine serum by western blot

In order to identify OvHV-2 antigens recognised by sera from animals with MCF, western blots of an OvHV-2 LGL-infected cell lysate using MCF-positive and negative clinical sera were performed using an OvHV-2 infected cell lysate. As a negative control, mock-infected BT cell lysate was blotted to determine the level of non-specific binding.

Two MCF-positive sera with different levels of antigen binding as determined by IFAT were tested (with weak and strong positive immunofluorescence detected) and shown to have the same pattern of reactivity against the OvHV-2 whole cell lysate (Figure 7.1). Four protein bands, estimated at 38, 48, 62 and 188 kDa were recognised by both MCF-positive sera. The MCF-negative clinical serum showed a different pattern of reactivity to the positive sera (Figure 7.1), with protein bands at 17, 28, 50 and 62kDa being recognised. Therefore the 62kDa is a non-specific protein, detected with both MCF-positive and negative sera. No reactivity was observed with the negative control mock-infected BT cell lysate. These initial findings suggested that sera from infected animals bound a set of specific proteins associated with the disease and indicated that MCF specific antigens might be identified by these sera.

In order to test the significance of these findings and to minimise any potential variation of antibody-antigen binding patterns between individual animals, a group of 20 clinical sera were tested. For the eighteen MCF-positive sera, a similar pattern of reactivity was seen in the majority of samples, with strong reactive protein bands at 17, 38, 48, 62, 140 and 188kDa, and weak bands at 28, 40 and 98kDa seen in some cases (Figure 7.2). This result differs from the banding pattern shown in the initial western blot with positive sera, as a larger number of proteins were observed overall. However there was a similarity in the reactivity pattern, suggesting that the difference in pattern may be a result of test sensitivity. This is supported by the fact that four of the six bands shown in the larger trial were seen in the preliminary test. Bands at 17 and 140kDa were not previously observed in MCF-positive serum samples (Figure 7.1). Other individual variations were also seen (Figure 7.2). Animal number 4, showed only two major protein bands, at high molecular weights of 50 and 65kDa. A second animal, number 11, showed only a weakly reacting band at

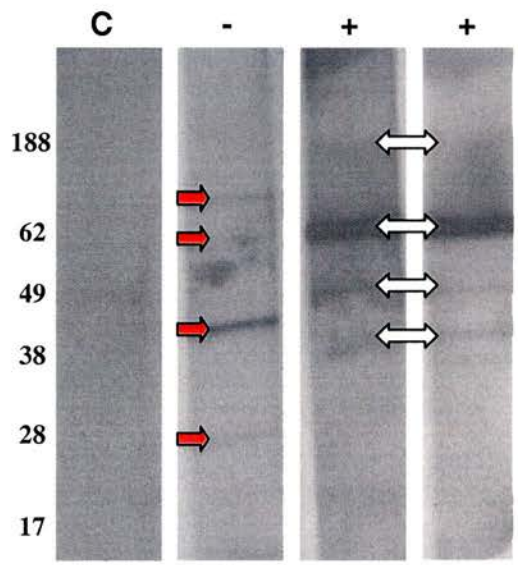


Figure 7.1: Chemiluminescent western blot of OvHV-2 crude lysate using MCF sera. Western blot using OvHV-2, BJ1035 whole cell lysate, with MCF positive (+) and negative (-) serum samples. As a negative control (represented by C), mock-infected BT cell lysate was blotted to determine the level of non-specific binding. The red arrows indicate protein bands present in the MCF-negative serum, while the white arrows indicate protein bands present in the MCF-positive serum.

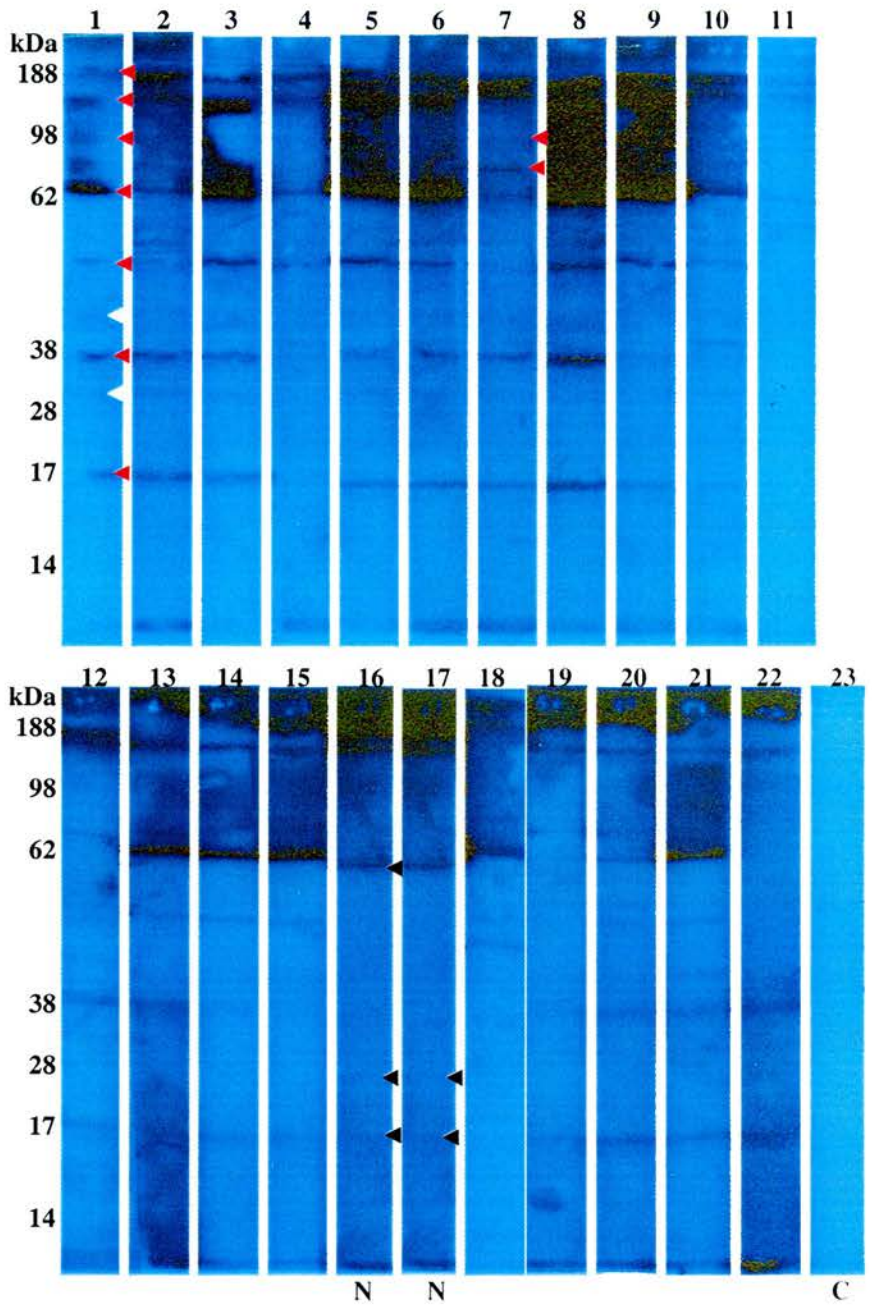


Figure 7.2: Chemiluminescent western blot of OvHV-2 crude lysate using MCF sera. Western blot using OvHV-2, BJ1035 whole cell lysate, with MCF positive and negative serum samples. C indicates secondary antibody used as a control (rabbit anti-bovine IgG). Red arrows indicate protein bands seen in the MCF-positive serum. Black arrows indicate protein bands seen in the MCF-negative serum (N).

65kDa, while animals 7 and 20 showed strong reactions to protein bands at molecular weights of 17 and 38kDa. Two animals, numbers 7 and 8, also showed a band at an estimated molecular weight of 100kDa, which was not detected by any other serum analysed. Of the two samples which were confirmed MCF-negative by IFAT (animal numbers 16 and 17), three protein bands, estimated at 17, 28 and 62kDa were recognised by both MCF-negative sera on analysis by western blot. As these bands were also recognised by MCF-positive sera, they were therefore eliminated as potential OvHV-2 antigens. As with the earlier findings, the secondary conjugated antibody showed no reactivity (lanes 11 and 12, Figure 7.1)

7.3.1.2. Comparison of clinical and experimental sera.

Serum from an immunised AlHV-1 (strain C500 high-pass) animal was compared to a clinical case of OvHV-2 MCF, confirmed by IFAT, in order to determine whether there was a difference in reactive proteins identified possibly due to the differences in infection, such as transmission, viral load and stage of infection. For the experimental animal, there is control over these aspects, while in naturally-infected field samples the precise onset and transmission of disease is unknown. Furthermore, conditions in which the clinical animals are exposed allow the possibility of other bacterial and viral infections.

The AlHV-1 strain WC11 was used as the source of virus antigen, being the antigen routinely used by IFAT and the WC11-ELISA (described in chapter five). The field sample showed a weak response, with bands present at 17, 36 and 48kDa and a double band at around 98kDa (Figure 7.3). In contrast, the experimental serum showed a strong response against the WC11 lysate, with bands observed between 28 and 188kDa, and three distinct bands at 36, 62 and 98kDa. Faint protein bands were also detected at 34, 48 and 188kDa.

7.3.1.3. Analysis of Immunized rabbit antisera by western blot

Serum samples from rabbits experimentally-infected with AlHV-1 and OvHV-2 (described in section 2.1.3) were analysed by western blot using an OvHV-2-infected LGL cell line whole cell lysate (Rosbottom *et al.*, 2002; Hart *et al.*, 2007), AlHV-1 strains WC11 and C500 cell lysates and a non-infected rabbit lymphocyte whole cell lysate (Figure 7.4).

The OvHV-2 rabbit antiserum is shown to react with more proteins in the OvHV-2 lysate than the AlHV-1 lysates. A strong pattern of reactivity was seen, with

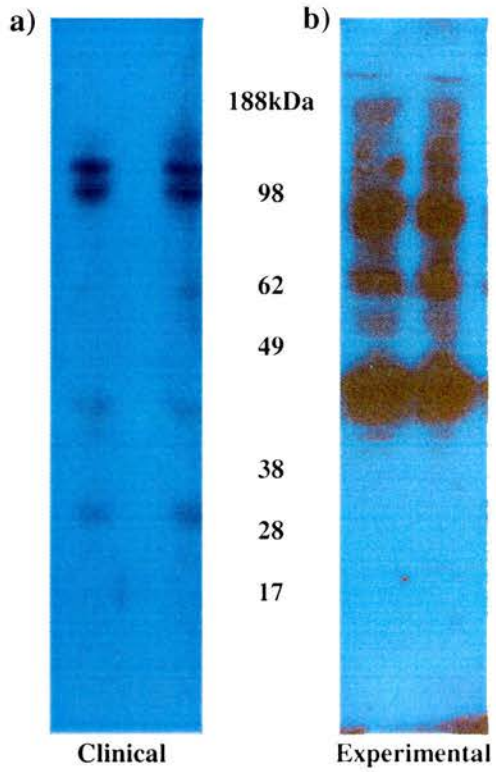


Figure 7.3: Chemiluminescent western blot of clinical OvHV-2 and experimentally infected AlHV-1 serum. a) Clinically-infected OvHV-2 bovine serum submitted for MCF testing and b) experimentally infected AlHV-1 MCF bovine serum, were analysed by western blot against MCF AlHV-1 strain WC11 cell lysate. Chemiluminescent blot performed using an HRP-labelled polyclonal rabbit anti-bovine conjugate.

distinct bands at 18, 28, 30, 36, 40, 45, 58, 64 and 80kDa (Figure 7.4a). The WC11 crude lysate showed distinct bands at 48, 64, 80, 175 and 180kDa. The C500 lysate also showed a distinct banding pattern, with a major band at 48 and 96kDa (Figure 7.4a). This result may imply that there is a lack of cross-reactivity between the anti-OvHV-2 serum and some AlHV-1 proteins. The AlHV-1 rabbit antiserum showed a similar pattern of reactivity with the majority of bands seen in all lysates at 50, 75, 80, 98 and 175kDa (Figure 7.4b). Additional bands were seen in the OvHV-2 lysate, at 30, 36 and 45kDa. The 45kDa was also seen in the WC11 lysate, but not the C500 lysate. The higher activity in AlHV-1 serum may be due to a higher viral load in infection.

Interestingly, both rabbit antisera showed a 54kDa band in the control rabbit lymphocyte lysate. Further bands seen in the control lane, include bands at 18, 80, 98 and 175kda, and suggest a degree of non-specific reaction possibly due to the conditions in which the virus inoculum and whole cell lysates are maintained. These proteins may therefore be excluded from further analysis as they indicate non-specific protein interaction.

7.3.1.4. Electrophoretic analysis of herpesvirus cross-reactivity

The lack of cross-reactivity between antibodies to bovine herpesvirus was previously analysed by the WC11-ELISA in chapter six (section 6.3.3). The specificity of the WC11-ELISA for antibodies against MCF antigens was confirmed in all cases. To identify MCF-specific virus proteins and conserved herpesvirus antigens, western blots were performed using infected cell lysates for AlHV-1 (WC11), OvHV-2, BHV-1, BHV-2, BoHV-4 and an uninfected BT cell control (described in section 2.2.2). The viral lysates were blotted with positive antiserum for each bovine herpesvirus as well as MCF-positive and negative antiserum from experimental animals (described in 5.2.3). The serum from an MCF-recovered animal (06-1176) was also used to analyse whether banding patterns from affected and recovered animals varied.

For the WC11 crude lysate, the bovine herpesvirus sera (BHV-1, 2 and 4) showed the same pattern of reactivity as the rabbit anti-bovine IgG control, with protein bands detected at 16 and 64kDa. This suggests the proteins recognised are conserved and may be immunoglobulins (Figure 7.5a). The MCF-positive serum showed a distinct pattern of six bands at 16, 40, 64, 98, 148 and 250kDa with weaker bands at 25, 55, 80 and 110kDa. The MCF-negative serum showed the same bands as

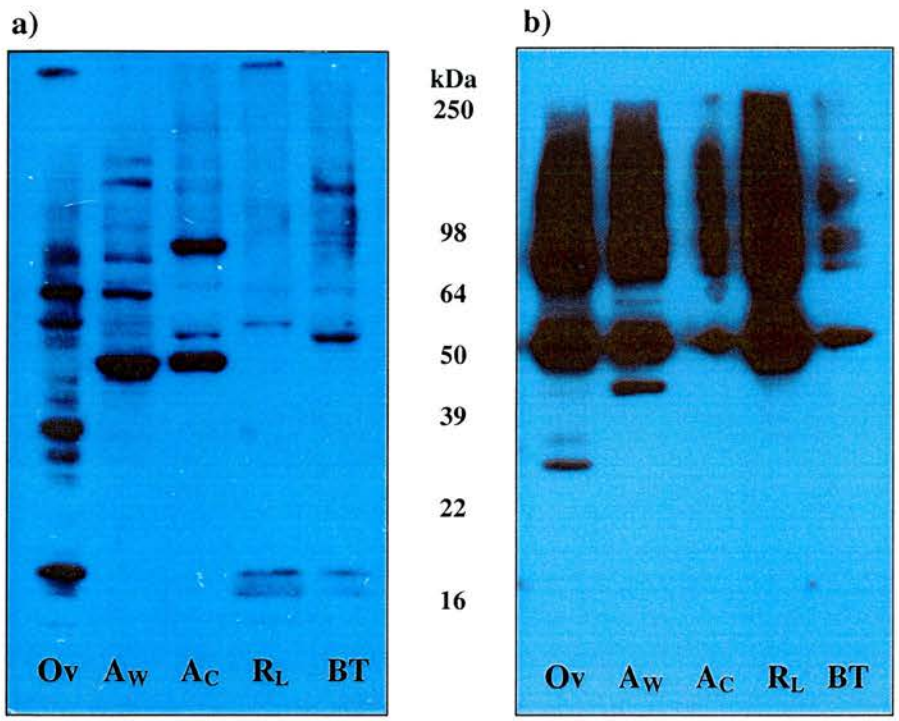
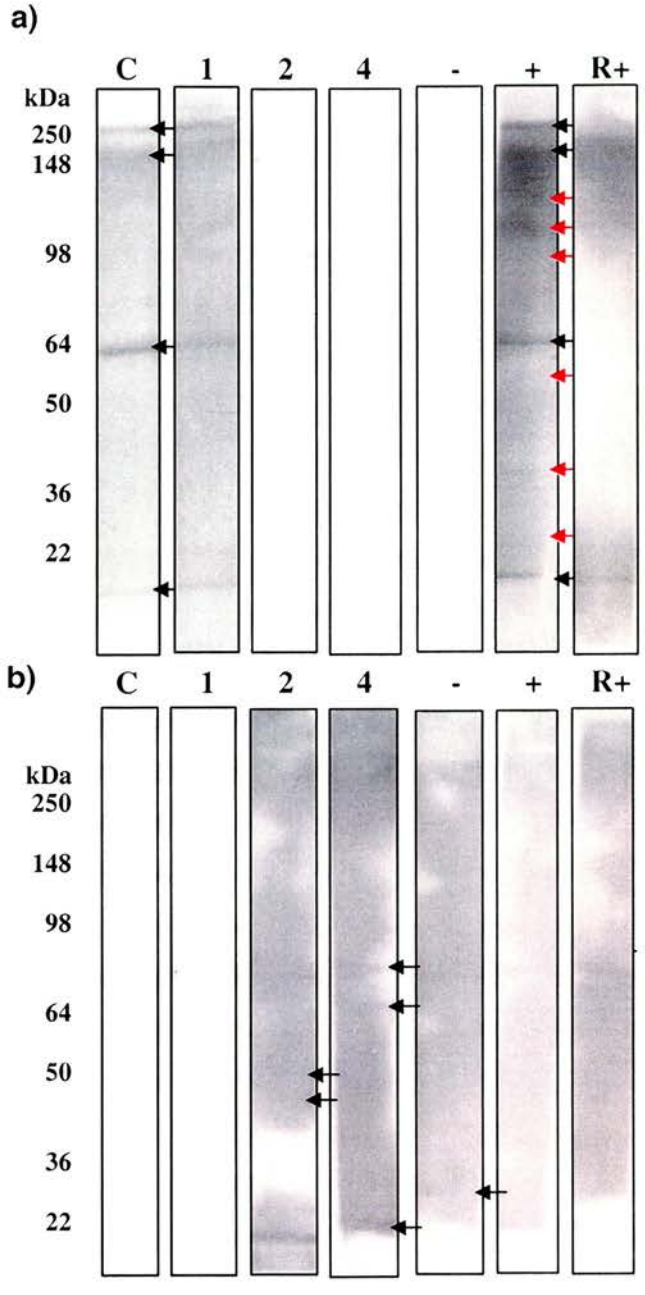


Figure 7.4: Analysis of experimentally-infected rabbit sera. Rabbits immunised with (a) OvHV-2 virus–infected mesenteric lymph node and (b) low-pass AlHV-1 strain C500, raised from liquid nitrogen and raised in bovine turbinate cells. Blood was collected on onset of clinical signs, were and serum was analysed using extracts from OvHV-2 (O_V), MCF AlHV-1 strains WC11 (A_W) and C500 (A_C), a rabbit lymphocyte cell (R_L) extract and BT lysate (BT) present as a control. Western blot performed using an HRP-labelled polyclonal goat anti-rabbit conjugate.

Figure 7.5: Chemiluminescent western blot to determine cross-reactivity herpesviruses. (**a**) WC11 and (**b**) OvHV-2 whole cell lysate were analysed with MCF positive (+) and negative (-) serum samples, a recovered clinical MCF serum sample (R+), BoHV-1 (1), BoHV-2 (2) and BoHV-4 (4). (C) Indicates a secondary anti-bovine IgG antibody control. The red arrows indicate protein bands unique to the MCF-positive serum, while the black arrows indicate protein bands conserved in all antisera tested.


a)
C
1
2
4
-
+
R+
kDa
250
148
98
64
50
36
22
b)
C
1
2
4
-
+
R+
kDa
250
148
98
64
50
36
22

seen for the IgG control and BoHV anti-serum (16 and 64kDa). The serum from an MCF-recovered animal was expected to show a similar banding pattern to that of the MCF-positive. However, only three bands were observed, two of which were the 16 and 64kDa bands seen in the control which appear to correspond to IgG heavy and light chains. The third band, at approximately 148kDa, was also seen in the positive serum sample, while the 40and 250kDa bands were absent. The lack of additional, MCF-specific, bands in the recovered serum may identify the 148kDa band as an important protective antigen.

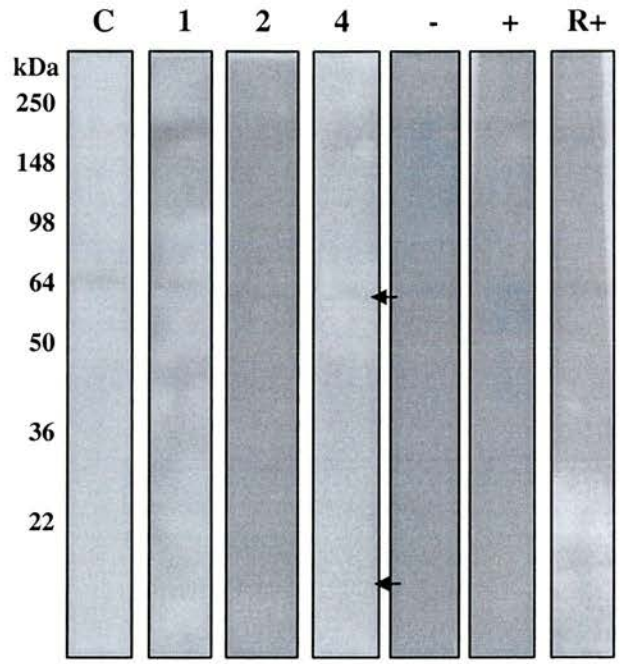
Analysis of the OvHV-2 whole cell lysate showed the MCF-negative serum sample and the IgG control showed a single band at 64kDa (Figure 7.5b). The MCF positive serum sample and the recovered clinical serum sample both show the same band at 64kDa, again probably corresponding to the IgG heavy chain. Faint protein bands are additionally seen at estimated molecular masses of 16, 45, 48, 60 and 68kDa. These bands are also found in all of the bovine herpesvirus antisera tested. The lack of unique bands reacting in the OvHV-2 whole cell lysate may be a result of the test sensitivity or poor expression of OvHV-2 antigens in these cells.

It was assumed the BT cell lysate would not react strongly with any of the antiserum samples tested, as this lysate was the basis for the control in the ELISA. However, as seen in the IgG control for the WC11-lysate, two bands were seen at 16 and 64kDa, corresponding in size to IgG heavy and light chains. A third band at approximately 250kDa was also seen in the BT cells with all antisera tested (Figure 7.5c). As the bovine cells are grown in bovine serum, bovine serum proteins may be present on both the polyacrylamide gel and blot, and may consequently be detected by the rabbit anti-bovine conjugate.

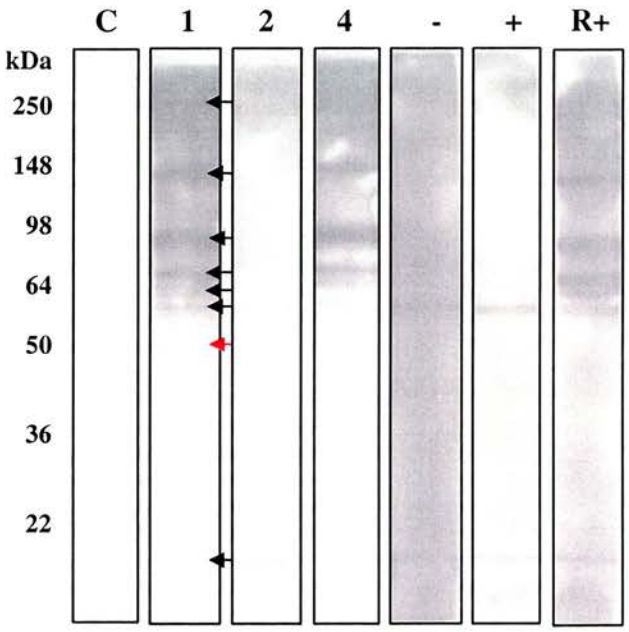
BHV-1 and BHV-2 have been shown not to cross-react with MCF viruses (Osorio *et al.*, 1985) and this finding was supported by results from the ELISA validation, where no serological cross-reactivity between MCF strain WC11 and BoHV-1 was shown. For the BHV-1 crude lysate (Figure 7.5d), the anti-bovine herpesvirus sera, BHV-1 and 4, all showed the same pattern of reactivity, with protein bands at 16, 50, 60, 64, 68, 98, 148 and 250kDa. The BHV-2, MCF-positive and negative sera detected bands at 16, 60 and 250kDa, all of which are bovine serum proteins recognised by rabbit anti-bovine conjugate. The MCF-recovered serum and the BoHV-4 antiserum showed the same pattern of reactivity as the BHV-1 antiserum, with the exception of a band at 50kDa. The 50kDa band was also detected by the MCF-positive serum. The secondary antibody control showed the

Figure 7.5: Chemiluminescent western blot to determine cross-reactivity herpesviruses. (**c**) Control BT lysate and (**d**) BoHV-1 whole cell lysate were analysed with MCF positive (+) and negative (-) serum samples, a recovered clinical MCF serum sample (R+), BoHV-1 (1), BoHV-2 (2) and BoHV-4 (4). (C) Indicates a secondary anti-bovine IgG antibody control. The red arrows indicate protein bands unique to bovine herpesvirus-1, while the black arrows indicate protein bands conserved in all antisera tested.

c)



d)



same two protein bands expressed in the WC11 lysate, corresponding to the IgG heavy and light chains at 16 and 64kDa (Figure 7.5a).

The BHV-2 crude lysate showed a distinct pattern with protein bands detected at 16, 64, 155 and 250kDa for all sera tested, with the exception of BHV-1 and BHV-2. BHV-1 antiserum only detected at 155 and 250kDa. The BHV-2 positive antiserum showed an additional band at approximately 68kDa (Figure 7.5e) that might therefore be BoHV-2 specific.

A western blot of the BoHV-4 crude lysate against the BoHV-4 positive antiserum shows bands at molecular weights of 16, 45, 64, 110, 148 and 250kDa (Figure 7.5f). This pattern of reactivity was conserved in all sera tested, with the exception of a band at 70kDa, only detected by BoHV-4 antiserum. The 70kDa may therefore be BoHV-4 specific. A band at 98kda was also detected using the MCF-positive antiserum, which was not detected with any other serum analysed. Furthermore the same bands were detected by the IgG control. This suggests that the BoHV-4 anti-serum cross-reacts with a number of bands detected by MCF antisera. These bands, which may be conserved among herpesviruses, may consequently be eliminated from further analysis for potential recombinant candidate proteins.

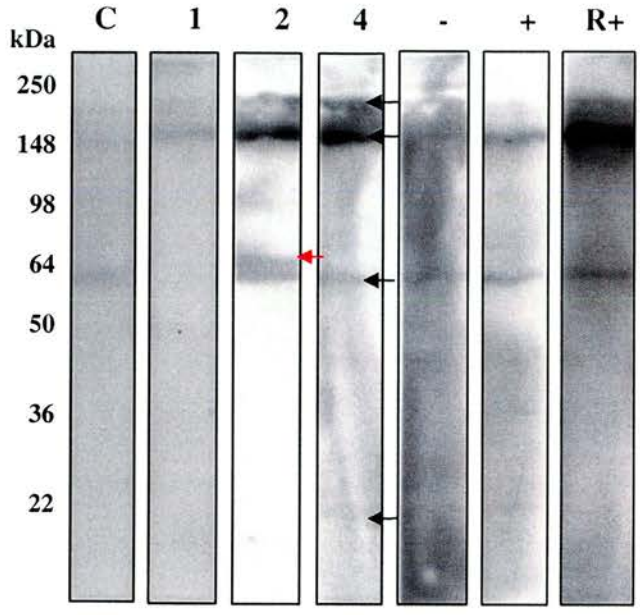
7.3.1.5. Electrophoretic analysis of ELISA lysate

To define the MCF antigens recognised in the ELISA, the crude ELISA lysate was analysed by staining with SimplyBlue SafeStain. Supernatant from the clarification centrifugation in the preparation of the ELISA antigen (5 minute clarification at 12,000 x g), the discarded cellular debris and subsequent ELISA antigen were compared to ELISA antigen prepared with C500, OvHV-2 and the control BT lysate (Figure 7.6a) by electrophoresis. Six distinct protein bands at 45, 55, 65, 97, 100 and 188kDa were seen in both the ELISA antigen and cell pellet and may be host proteins. These bands were extracted and analysed by MALDI mass spectrometry. Four bands were observed in the cell pellet at 15, 17, 18 and 19kDa which were not seen in the ELISA antigen. It is possible these proteins were present in the ELISA antigen at a lower concentration and were therefore not detected using SimplyBlue stain. These bands were additionally sent for MS analysis.

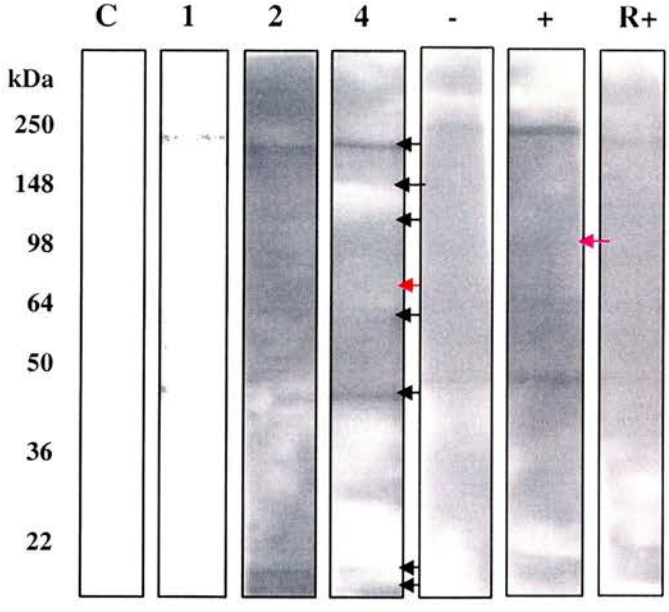
Analysis by western blotting (Figure 7.6b), using MCF-positive serum, showed two distinct bands expressed in the ELISA antigen at 64kDa (Figure 7.7 band 6) and 148kDa (Figure 7.7 band 3), with faint bands at 20kDa (Figure 7.7 band 10) and 50kDa (Figure 7.7 band 7). The cell pellet showed the same pattern of

Figure 7.5: Chemiluminescent western blot to determine cross-reactivity herpesviruses. (**e**) BoHV-2 and (**f**) BoHV-4 whole cell lysate were analysed with MCF positive (+) and negative (-) serum samples, a recovered clinical MCF serum sample (R+), BoHV-1 (1), BoHV-2 (2) and BoHV-4 (4). (C) Indicates a secondary anti-bovine IgG antibody control. The red arrows indicate protein bands unique to the bovine herpesvirus, black arrows indicate protein bands conserved in all antisera tested. Pink arrows indicate a band unique to the MCF-positive serum.

e)



f)



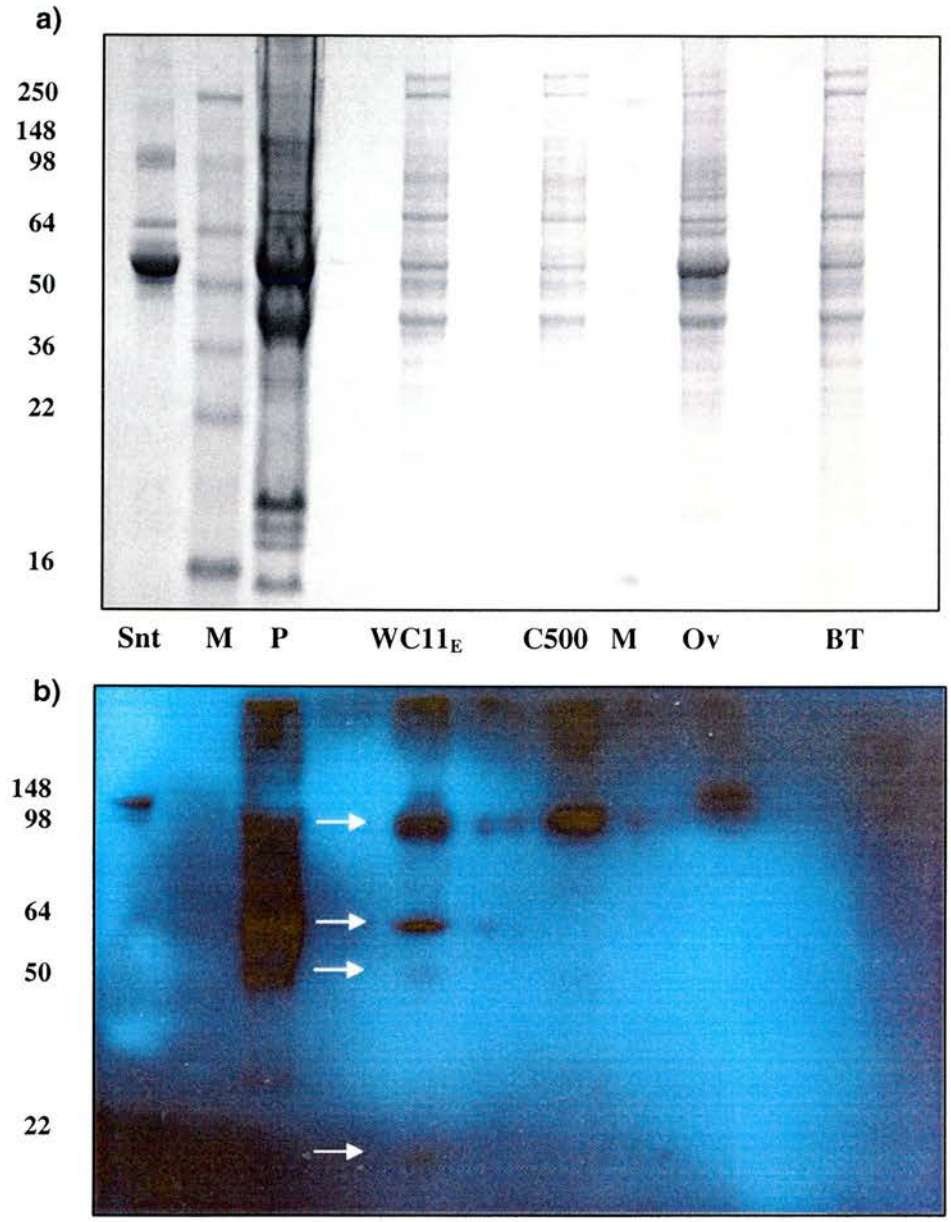


Figure 7.6: SDS-PAGE analysis of ELISA antigen. The clarified supernatant (Snt), cell pellet (P) and the ELISA antigen ($WC11_E$) were compared to crude C500, OvHV-2 and control BT lysates. Lysates resuspended in 2× SDS loading buffer, run at 200v on a 12% tris-glycine polyacrylamide gel. Gels were either stained with SimplyBlue SafeStain (a) or analysed by western blot using MCF-positive sera (b). SeeBlue Plus2 Prestained protein marker was used as size standard (250-6kDa). Protein bands in the ELISA antigen shown by arrows.

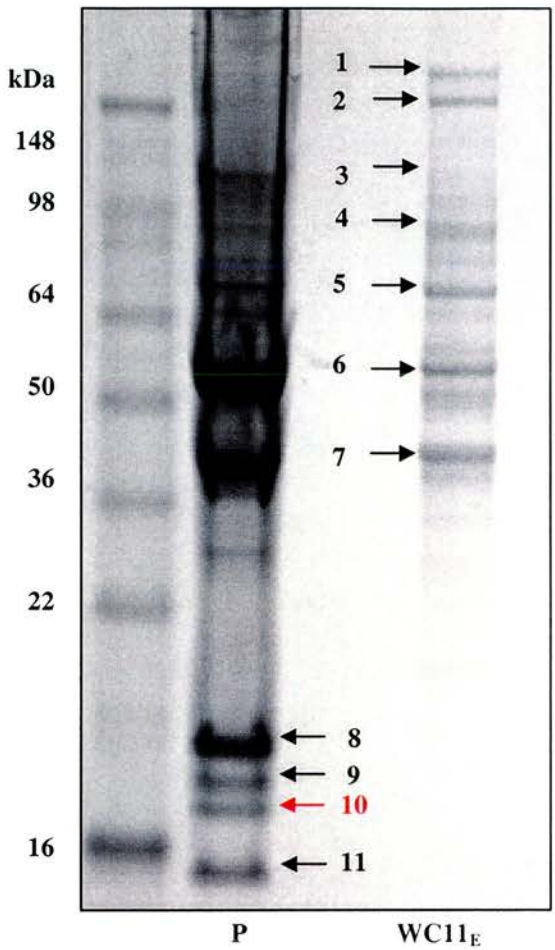
reactivity, with additional bands at 98kDa (band 5) and120kDa (Figure 7.7 band 4). Analysis of the AlHV-1 strain C500 and OvHV-2 BJ1035 lysate showed a single band at approximately 148kDa. No bands were seen in the BT lysate control.

Proteomic analysis showed that most of the major bands in SimplyBlue stained gels were host proteins (Figure 7.7). One protein (band 10) was shown to have 51% sequence coverage for AlHV-1 ORF52 identified from a band present in the cell pellet at 17kDa. OvHV-2 ORF52 encodes a 136 amino acid (16kDa), homologous to HSV UL49 which encodes VP22. This is one of the components of the tegument although its role in viral infection has not been defined. UL49 is conserved among all alphaherpesviruses including bovine herpesvirus-1. A band of the expected molecular mass was subsequently shown in the ELISA antigen by western blot, using MCF-positive serum. Therefore the protein was possibly masked by the presence of IgG in the serum.

7.3.2. Analysis of AlHV-1 antigen using affinity chromatography

As the experimental bovine serum (tested in 7.2.1.2) showed high reactivity to AlHV-1 and OvHV-2 lysates, alternative methods were sought to identify candidate proteins by purification or fractionation of potential MCF antigens present in the lysate. Affinity chromatography was used to enrich antigen-specific antibodies from anti-MCF serum (described in section 7.2.3).

Protein-A purified AlHV-1 serum was used in a western blot against AlHV-1 WC11 and OvHV-2 BJ1035 crude infected cell lysate. Purification of the MCF AlHV-1 antiserum appeared successful as less background interference was seen than previously observed when using crude sera (Figure 7.8). There were slight differences observed between the proteins detected on the two MCF lysates. Five distinct protein bands at 30, 34, 64, 130 and 250kDa (Figure 7.8a) were recognised in the AlHV-1 lysate. The purified IgG recognised three bands in the OvHV-2 lysate (34, 64 and 250kDa) which were also present in AlHV-1 as well as additional bands with molecular weights of 36, 46, 48, 52 and 80kDa (Figure 7.4b). As the serum has been purified using the WC11 lysate affinity column it is possible that only antibodies which interact AlHV-1 antigens would be bound to the column.



Band	Proteomic Hit
2	Myosin
3	Actin Isoform
4	Glucose regulated protein precursor (GRP 78)
5	BSA
6	Not Identified
7	Actin
8	Histone
9	Histone
10	AlHV-1 orf52
11	Histone

Figure 7.7: WC11-ELISA antigen analysed by Proteomic MALDI mass spectroscopy. Distinct bands from the cell pellet (P) and the ELISA antigen ($WC11_E$), numbered 1-11, were analysed by MALDI mass spectrometry. Band one was not analysed.

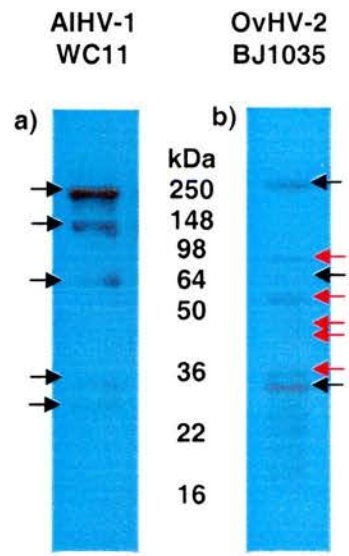


Figure 7.8: Chemiluminescent western blot of MCF-positive serum. MCF serum taken from an animal experimentally infected with AlHV-1 was purified using an AlHV-1 affinity purification column. Purified serum was analysed by western blot using AlHV-1 WC11 crude lysate (a) and OvHV-2 BJ1035 crude infected cell lysate (b). Arrows (black) indicate the major bands observed while red arrows indicate bands only seen in the OvHV-2 lysate.

7.3.2.1. Affinity chromatography using IgG purified serum

A second affinity purification column was prepared with IgG purified, using HiTrap protein-A, from MCF-positive serum in order to directly bind specific antigens. Purified bovine IgG (5ml 325µg/ml) was dialysed against coupling buffer (0.1M $NaHPO_4$, 0.5M NaCl, pH 8.3) prior to injecting on to the column. WC11 lysate (5mls 589µg/ml) was loaded onto the column. The chromatogram showed a single peak (not shown) containing 55µg of eluted protein (fractions 14-16). The fractions were concentrated 30-fold and analysed by electrophoresis and western blot (Figure 7.9). No protein was detected in the three fractions when stained with SimplyBlue safestain, suggesting the protein was lost during concentration. Alternatively, this result may also indicate that there was a high level of non-specific antigens which may be masking the MCF specific antibodies. Furthermore the antigens specific to the bovine sera may be at a low concentration in the lysate and are therefore not detected. To increase the sensitivity of detection, concentrated fractions were pooled and electrophoresed. The gel was then stained using Sypro Ruby stain which can detect 1ng of protein per band. Similar to findings with SimplyBlue SafeStain, no protein was detected in the fractions, however protein was again observed in the controls and fall-through material (Figure 7.10). This again suggests that the protein was lost during concentration.

The lack of protein bands observed in the eluted fractions by two sensitive staining methods suggests that the protein content was insufficient. This may suggest that there were not enough specific antibodies loaded onto the column resulting in a low binding capacity. The amount of MCF-specific antibody bound to the column is unknown. A second explanation for the lack of protein may be a result of the detergent used in the preparation of the ELISA antigen, inhibiting binding.

The experiment was repeated, using a second column prepared with Protein-A purified AlHV-1 MCF-positive serum. To check that the Ig bound to the column, an ELISA was performed on Ig samples before and after binding to the column (Figure 7.11). There was a noticeable difference between crude (0.9AU) and unbound (0.26AU) samples, where there was a difference of 0.65 absorbance units, about a third of the initial absorbance value present in the unbound sample. This suggests that the column had bound most of the antibody. However, one consideration that must be made is to the dilution of the unbound serum sample in comparison to the crude serum.

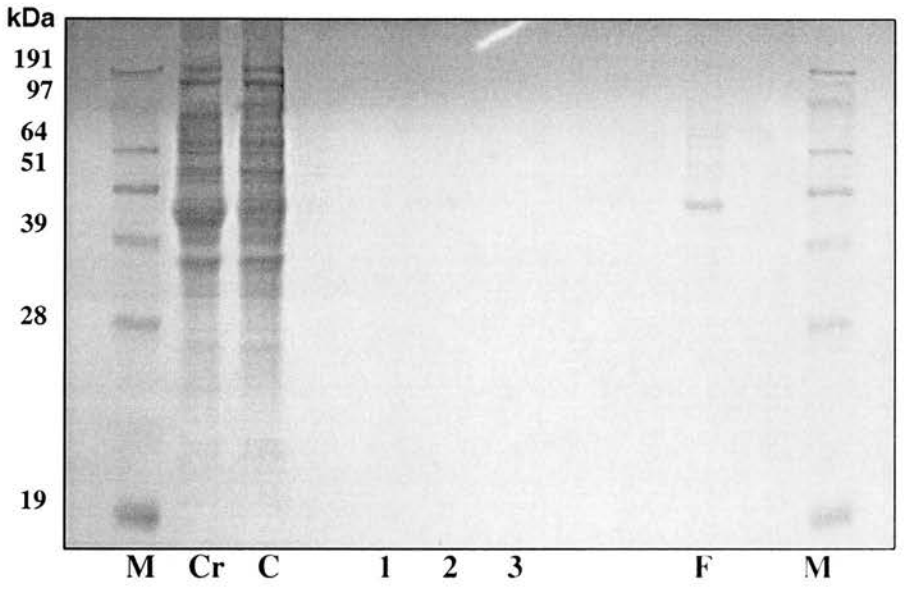


Figure 7.9: SDS-PAGE analysis of fractions from an anti-MCF serum affinity purification (NHS-HiTrap) column. Cr represents crude WC11 lysate. C represents BT cell lysate control and F represents column fall-through material. Crude AlHV-1 WC11 lysate (5mls 589µg/ml) diluted ten-fold with PBS was loaded onto the column. Thirty 1ml fractions were collected where activity was shown o the chromatogram as a single peak, contained in three 1ml fractions (Fraction 14 -**1**, Fraction 15 -**2**, Fraction 16 -**3**). The three fractions were concentrated 5-fold and 25µl was loaded on a 14% tris-glycine polyacrylamide gel. The gel was analysed by gel staining SimplyBlue SafeStain. No protein was detected by western blot (not shown), using MCF-positive sera and a rabbit anti-bovine (IgG) HRP conjugate.

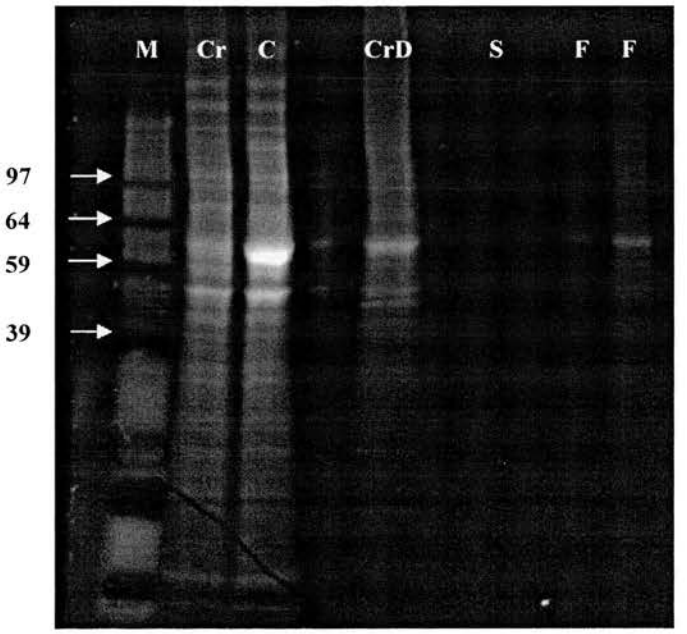


Figure 7.10: SDS-PAGE analysis of AlHV-1 fractions taken from an anti-MCF serum affinity purification (NHS-HiTrap) column. Cr represents crude WC11 lysate, C represents BT cell lysate control, CrD represents crude WC11 lysate following dialysis and F represents column fall-through material. Crude AlHV-1 WC11 lysate (5mls 589μg/ml) diluted ten-fold with PBS was loaded onto the column. Thirty 1ml fractions were collected and three fractions were concentrated 30-fold (S). Twenty-five microlitres of pooled fraction sample was loaded on a 14% tris-glycine polyacrylamide gel, were analysed by gel staining with Sypro Ruby stain.

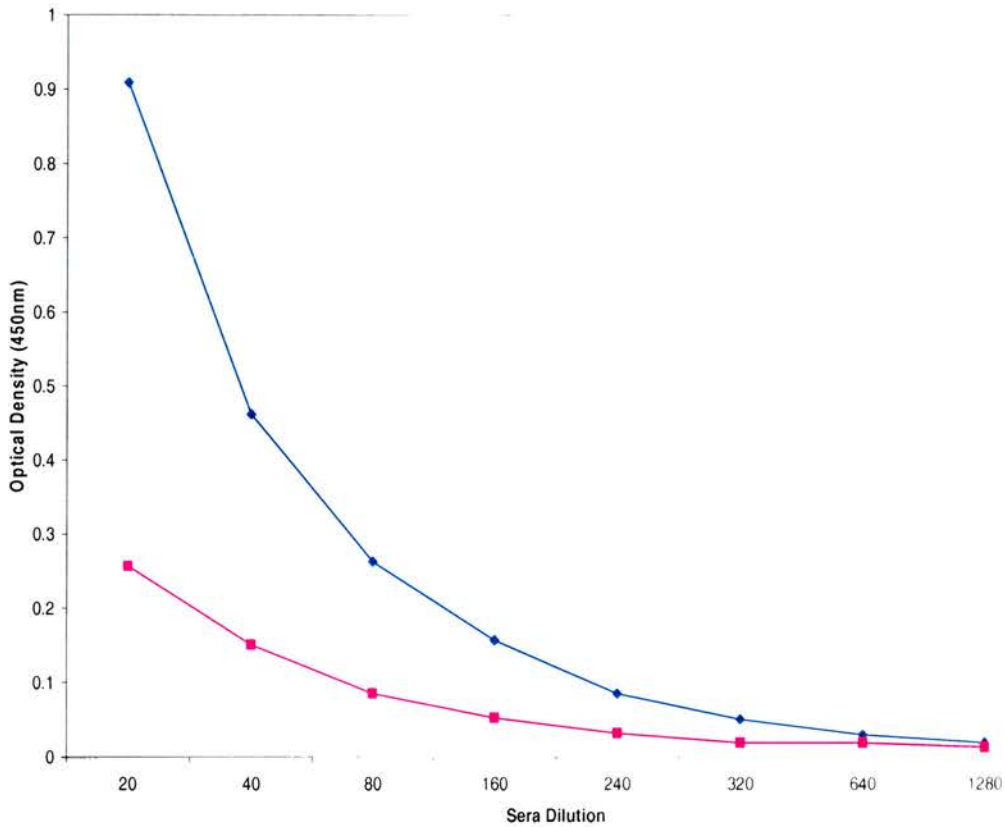


Figure 7.11: Analysis of HiTrap column binding efficiency. HiTrap Protein-A purified AlHV-1 MCF-positive serum was analysed using the MCF WC11-ELISA. Samples of crude and sample which remained unbound to the HiTrap Protein-A affinity column, were analysed at a range of serial dilutions (1/20 – 1/1280). Optical density was measured at 450nm. Dilutions were analysed in duplicate.

Having checked the binding capacity of the column, crude WC11 lysate (1.5mg/ml), diluted 10-fold in PBS, was injected onto the column using the FPLC system. The unbound fraction was washed off the column and the bound protein was eluted in a single fraction containing 16μg of protein.

To investigate whether there was a difference in the antigenic proteins expressed within the two related viruses, OvHV-2 crude lysate (0.48mg/ml) was injected on to the column (5mls diluted 10-fold in PBS) and a single fraction containing 15μg bound antigen was eluted. Both AlHV-1 and OvHV-2 eluted fractions were concentrated 10-fold and analysed by SDS-PAGE gel electrophoresis and western blotting. No bands were detected in either lysate eluant when stained (Figure 7.12). Proteins with molecular masses ranging from 191 to 39kDa were seen in the unbound cell lysate, with a major band at 64kDa. This band was also seen in the unbound material. Western blotting using experimental serum (described in section 7.2.1.2) showed a faint band in the eluant, with a molecular mass of 55kDa. No bands were detected in the unbound cell lysate (Figure 7.12).

As no protein was detected by SDS-PAGE electrophoresis, work on affinity purification methods was not continued. A possible explanation for the failure to identify MCF specific antigens by this method may be the presence of the non-ionic detergent used to prepare the ELISA antigen (NP40) inhibiting active binding to the column.

7.3.3. Fractionation of MCFV lysates

Further purification methods were sought for the identification of antigens recognised by the ELISA that were more compatible with the ELISA antigen preparation. Fractionation of the ELISA lysate to isolate proteins of interest was performed by gel filtration in order to simplify the crude lysate protein mixture. Crude WC11 lysate (0.5mg/ml) was injected into a Superdex200 gel filtration column using an FPLC system. Thirty-five (1ml) fractions were collected and fractions which corresponded to peaks on the FPLC system UV trace were analysed by Coomassie staining and western blot (Figure 7.13). The analysis was repeated for a BT control lysate (Figure 7.14)

For the WC11 crude lysate, stained gels showed protein in only the initial three protein peaks, with proteins ranging from 28 to 100kDa. Fraction 5, which corresponded to the highest peak from the fractionation trace (Fractions 5-7), showed a smear of detected bands (Figure 7.13b). Fractions 6 and 7 also taken from the

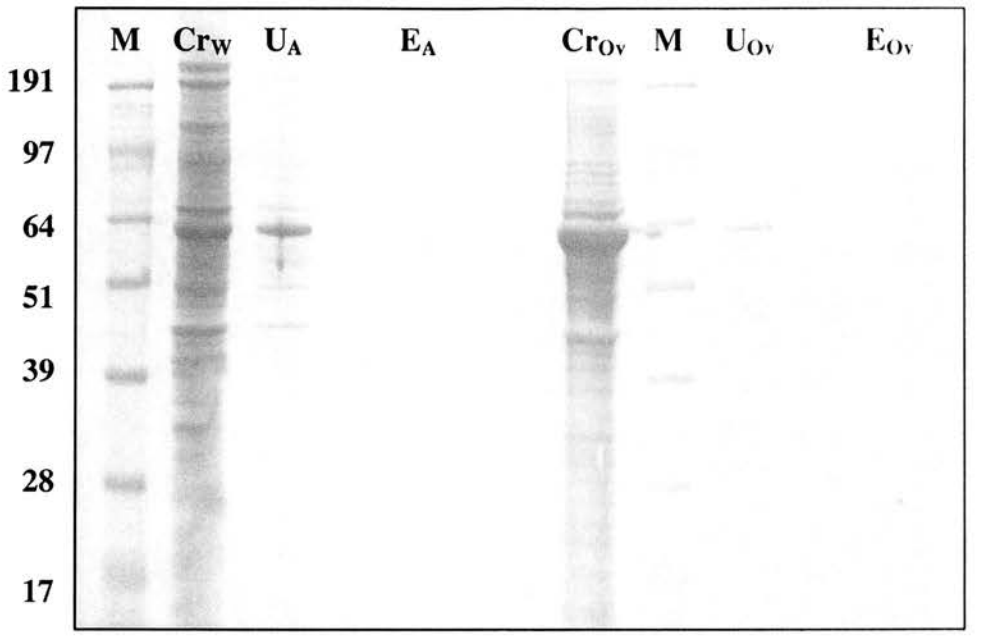


Figure 7.12: SDS-PAGE analysis of AlHV-1 and OvHV-2 lysates purified using an anti-MCF serum affinity column (NHS-HiTrap). Cr represents crude WC11 lysate (Cr_w) and BJ1035 crude infected cell lysate (Cr_{ov}), U represents unbound material and E represents eluted material from the anti-MCF serum affinity column, for both AlHV-1 (E_A) and OvHV-2 (E_{Ov}). Gel stained with SimplyBlue Safestain.

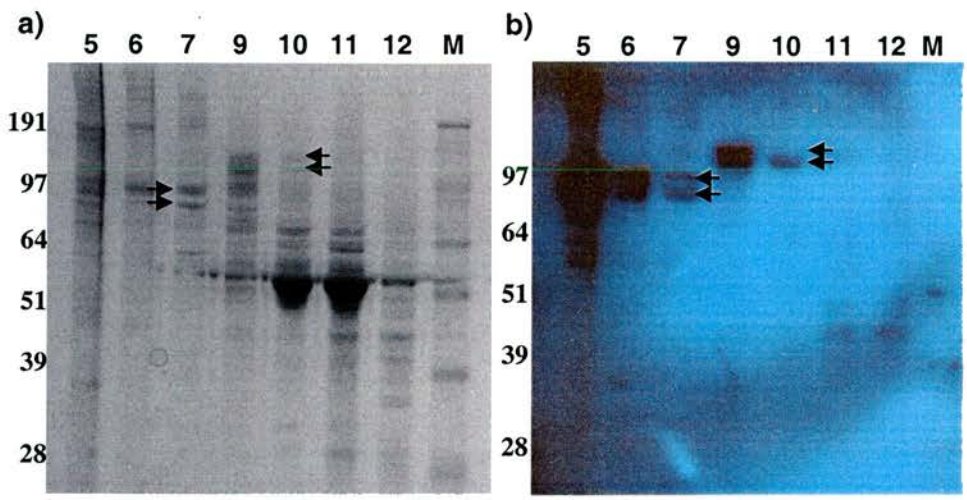


Figure 7.13: SDS-PAGE analysis of AlHV-1 WC11 lysate purified by gel filtration. Using the Akta FPLC system, fractions showing any presence of protein by OD were analysed by electrophoresis (a) and western blot (b) using MCF antisera. A 14% tris-glycine polyacrylamide stained gel shows fractions corresponding to highest activity on the chromatogram. Arrows on stained gel represent bands recognised by MCF antisera by chemiluminescent western blot.

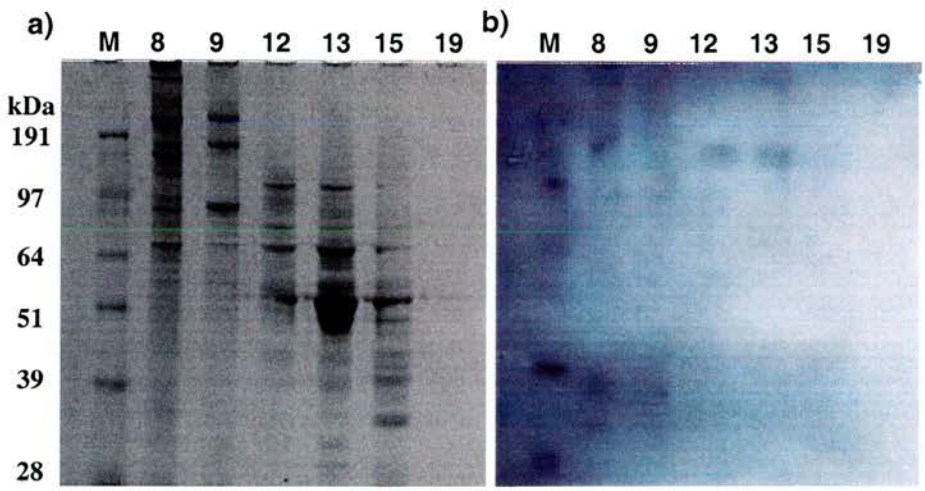


Figure 7.14: SDS-PAGE analysis of control lysate (BT) purified by gel filtration. Using the Akta FPLC system, BT lysate fractions showing any presence of protein by OD were analysed by electrophoresis (a) and western blot (b) using MCF antisera. A 14% tris-glycine polyacrylamide stained gel shows fractions corresponding to highest activity on the chromatogram.

initial fractionation peak showed less protein than fraction 5. Fractions corresponding to the second peak (Fractions 9-11) showed distinct protein bands at 45, 48, 60, 64, 66 and 70kDa. Fraction 12, which corresponded to the third peak, showed a similar banding pattern to the previous fractions with bands at 36, 40, 45, 48, 55, 60, 64, 66 and 70kDa. The strong band at 60kDa shown by staining was thought to be bovine serum albumin. By western blot, using MCF positive antisera (Figure 7.13b), fraction 5 showed a smear of detected bands with a distinct band at 97kDa, while fractions 6 and 7 showed only a triple band at 97kDa. Fractions 9 and 10 showed bands around 110kDa, while the third protein peak showed no distinct proteins recognised by MCF antisera. Although a high number of protein bands were visible by staining, no other antigenic bands were detected by the positive MCF crude serum.

For the BT crude lysate the chromatography trace showed the same protein peaks and therefore the corresponding fractions were analysed. Similar to the WC11 fractionation, the stained gels showed detectable protein bands in only the initial three peaks, with proteins ranging from 28 to 100kDa (Figure 7.14a). No bands were observed by western blotting using positive MCF crude serum (Figure 7.14b). This suggests that proteins detected in the WC11 lysate were specific to MCF.

Proteomic analysis was performed on the bands at 97kDa recognised by MCF positive sera (Figures 7.15). The only sequence match identified bovine serum albumin. This suggests that the high level of serum albumin present in the lysate may mask the identification of AlHV-1 viral proteins.

7.3.3.1. Purification of WC11 crude lysate using Ion Exchange

A further purification step was performed, to reduce background interference seen in the fractionated samples, using ion-exchange with Q-sepharose columns. A pool (1ml) of fractions 5, 6 and7, taken from the initial peak of the WC11 crude lysate, was diluted in binding buffer (20mM Tris pH8.2) and injected onto a Q-sepharose column using an FPLC system. Forty-five (1ml) fractions were eluted using a linear salt gradient to 1M NaCl and fractions containing protein, by absorbance at 280nm, were concentrated 10-fold, resuspended in SDS loading buffer and analysed by staining and western blot (Figure 7.16). A control was also tested, using the corresponding samples from the BT crude lysate fractionation (fractions 8-10).

By staining, protein could be detected in the initial peaks taken from the salt gradient, ranging in size from 50 to 191kDa. There was some background smearing

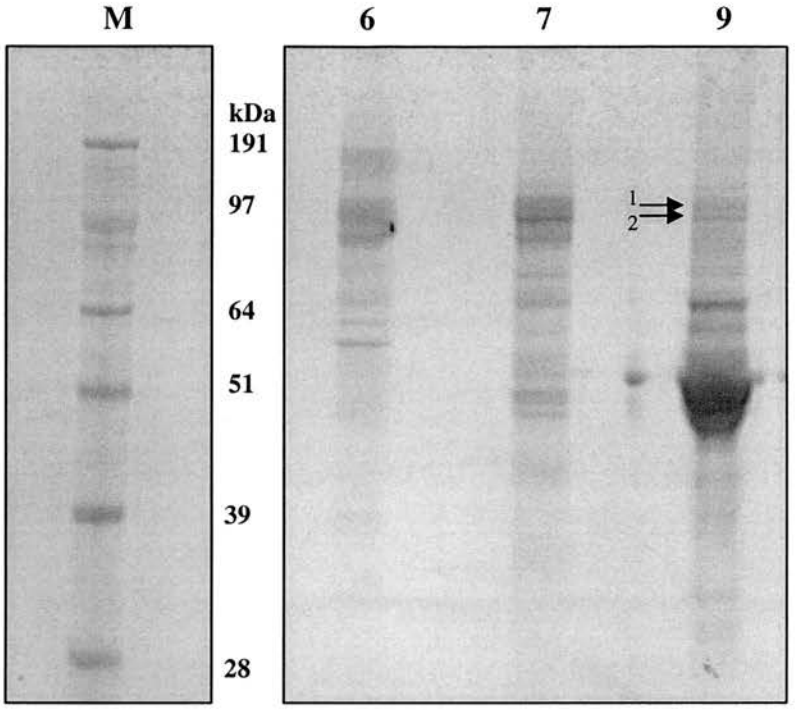


Figure 7.15: AIHV-1 gel purified proteins analysed by Proteomic MALDI mass spectroscopy. Proteomic analysis was performed on the bands at 97kDa, recognised by MCF positive sera (Figures 7.13). Fractions run on a 12% bis-tris polyacrylamide gel and stained with SimplyBlue Safestain, were analysed by MALDI mass spectrometry. Arrows indicate two bands analysed (97kDa).

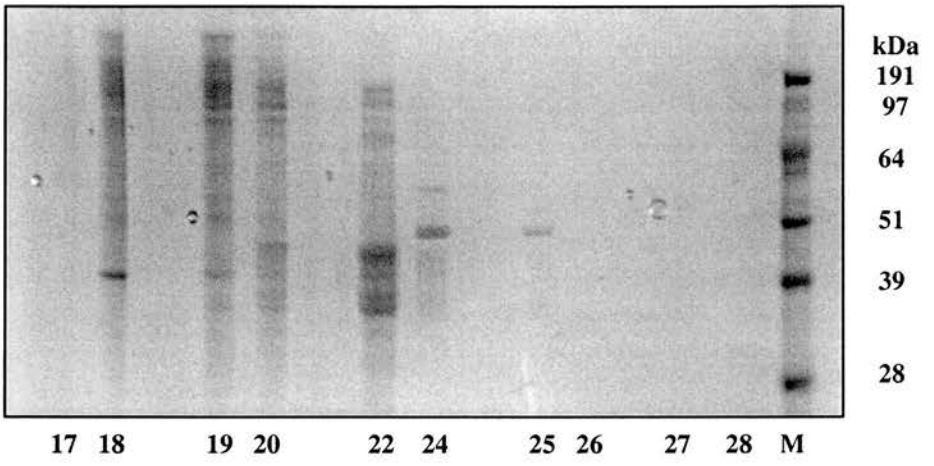


Figure 7.16: AlHV-1 fractions purified using a Q-Sepharose column. A pool of fractions (fractions 5-7) taken from AlHV-1 lysate, previously purified by gel filtration were further subjected to purification using a salt-gradient Q-Sepharose column. Forty-five 1ml fractions were eluted from the column and fractions showing any presence of protein by OD were concentrated 10-fold and analysed by SDS-PAGE.

on the gel, resulting in difficult interpretation between individual bands. Fraction 22 showed three distinct bands at 30, 40 and 45kDa, while fractions 24 and 25 showed a single protein band at 50kDa. No antigenic proteins were detected by western blot. This was possibly due to the use of the detergent NP-40 to lyse the WC11 infected BT cells. NP-40 can interfere with the binding of proteins to columns because of its low micelle size and strong binding properties. It is therefore possible that the antigenic proteins remained in the unbound fraction. The experiment was repeated using the collected unbound fraction, no protein was eluted using the Q-sepharose column, and therefore no analysis was performed on this fraction.

7.3.3.2. Fractionation of OvHV-2 BJ1035 ELISA antigen lysate

Fractionation of the OvHV-2 ELISA lysate to isolate specific proteins was also performed. BJ1035 lysate (0.5ml; 0.48mg/ml) was injected into a $Superdex_{200}$ fractionation column using an Akta FPLC system. Twenty-five (1ml) fractions were collected and fractions corresponding to peaks on the FPLC system trace were analysed by Coomassie staining and western blot (Figure 7.17). Analysis by stained gel showed low levels of protein, with faint bands at 52, 55 and 64kDa (Figure 7.17a) observed in fractions from the second protein peak (fractions 12-15). By western blot, only one strong protein band at about 97kDa reacted with the MCF specific serum (Figure 7.17b). Although this was the only protein shown on the western blot, additional protein bands were seen at the top of the gel, possibly indicating the presence of insoluble or very high molecular weight protein complexes.

Proteomic analysis was performed on the 97kDa band recognised by MCF positive serum, identified in OvHV-2 fraction 11. Identification of the protein proved unsuccessful as the only sequence match identified an IgG-associated protein (Figures 7.18). This would suggest that contamination by host proteins masked the presence OvHV-2 antigens.

7.3.4. CHAPS lysed AlHV-1 lysate

To determine whether inhibition by NP-40 was observed in the affinity purification, lysis of the viral pellet was performed using a variety of detergents: Triton X-100 (non ionic); CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate), a high ionic strength buffer (NaCl/TNE); and sodium dodecyl

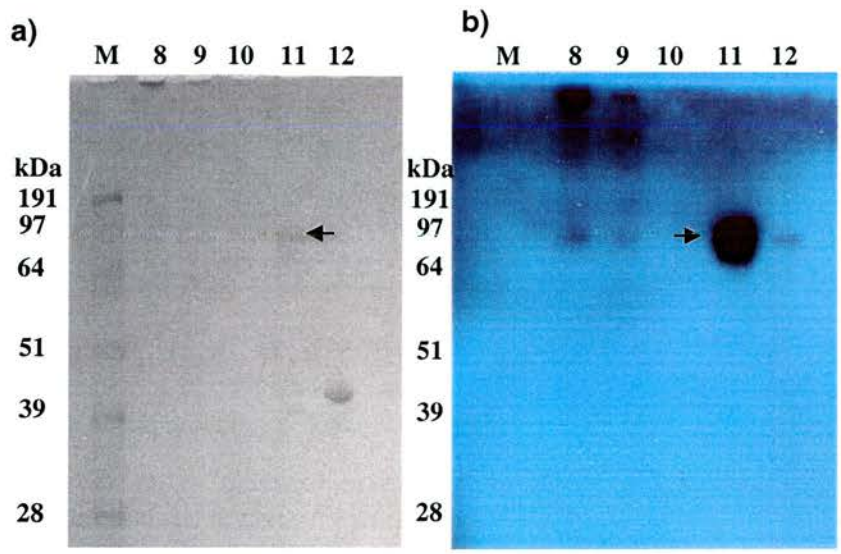


Figure 7.17: SDS-PAGE analysis of OvHV-2 lysate purified by gel filtration. Using the Akta FPLC system, fractions showing any presence of protein by OD were concentrated 10-fold and analysed by electrophoresis (a) and western blot (b) using anti-MCF serum. A stained 14% tris-glycine polyacrylamide gel shows fractions corresponding to highest OD on the chromatogram. Arrows on stained gel represent bands recognised by MCF antisera by chemiluminescent western blot.

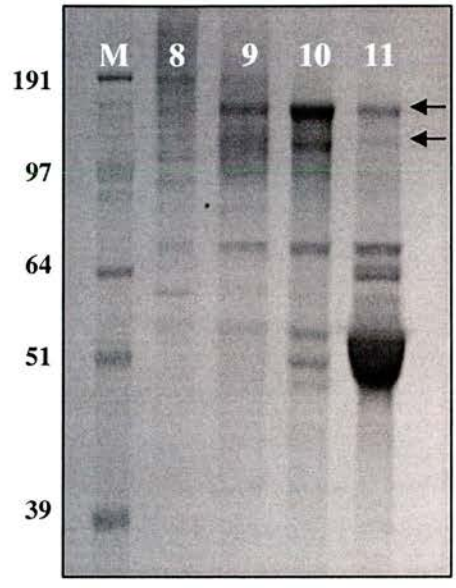


Figure 7.18: OvHV-2 gel purified proteins analysed by Proteomic MALDI mass spectroscopy. Proteomic analysis was performed on bands recognised by MCF positive sera (Figures 7.17). Concentrated ractions run on a 12% bis-tris polyacrylamide gel and stained with SimplyBlue Safestain, were analysed by MALDI mass spectrometry. Arrows indicate two bands analysed.

sulphate (anionic) (Figure 7.19). Four major bands were identified in each extraction lysate at 32, 35, 43 and 45kDa. Extraction by CHAPS and the high ionic strength buffer, when compared to the remaining lysates, showed the clearest banding pattern with additional bands observed at 58, 62 and 66kDa.

Analysis of the CHAPS and NP-40 extracted lysates by western blotting (Figure 7.19b), using anti-MCF serum, showed distinct bands expressed in the WC11 pellet at 32, 35, 43 and 55kDa. As seen by staining, extraction by CHAPS showed a stronger response with additional bands observed at 45, 50, 60 and 66kDa. These bands were not seen in the NP-40 extract.

As CHAPS is an ionic detergent, it may not inhibit the binding of proteins to an affinity or fractionation column. Therefore the CHAPS lysed cell-free virus pellet was further analysed by SDS-PAGE gel electrophoresis (Figure 7.20). Furthermore, to reduce contaminants observed in previous proteomic analysis, the WC11 virus for this experiment was grown in serum-free medium, rather than in the presence of 2% foetal calf serum. Four distinct protein bands could be discerned at 48, 66, and a double band at 130kDa. Faint bands were also shown at 36, 38 and 40kDa.

Proteomic analysis was performed to identify the four major bands shown by the SimplyBlue stained gel (Figure 7.21). All bands had positive sequence matches to the AlHV-1 genome. Bands 1 and 2, with estimated molecular weighs of 130kDa, identified AlHV-1 ORF25, with 31% and 22% sequence coverage respectively. Band 3, estimated molecular weight of 66kDa, identified AlHV-1 ORF21 showing 16% sequence coverage. Band 4, estimated molecular weight of 45kDa, initially showed a positive hit to actin (bovine), which is one of the most highly conserved proteins. When analysed in the absence of actin, AlHV-1 ORF54 was identified, with 18% sequence coverage. AlHV-1 ORF21 encodes a 569 amino acid protein, thymidine kinase, homologous to HVS ORF21 (26%) and EBV BXLF1 (29%). AlHV-1 ORF25 encodes the 1370 amino acid major capsid protein. AlHV-1 ORF25 is homologous to HVS ORF25 (56%), EBV BCLF1 (54%). AlHV-1 ORF54 encodes a 298 amino acid protein with homology to dUTPase (deoxyribouridine–triphosphate pyrophosphatase), an enzyme involved in viral replication and which is found in the virus particle (Chen *et al.*, 2002). AlHV-1 ORF54 is homologous to HVS ORF54 (36%), EBV BLLF1 (27%).

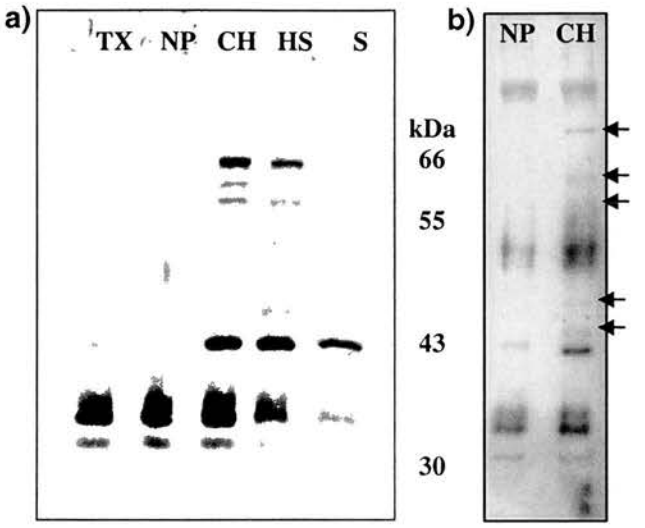


Figure 7.19: Comparison of AlHV-1 WC11 virus pellet extraction methods. a) Five extraction methods were compared using WC11 cell-free virus. Different extraction methods include the use of detergents, Triton X-100 (TX), NP-40 (NP), 0.08% CHAPS (CH), High-salt buffer (HS) and SDS loading buffer (SDS). The CHAPS extracted AlHV-1 WC11 pellet showed the strongest banding pattern and was analysed against NP-40 by western blot (b), using anti-MCF serum. Arrows indicate bands not shown in the NP-40 extract.

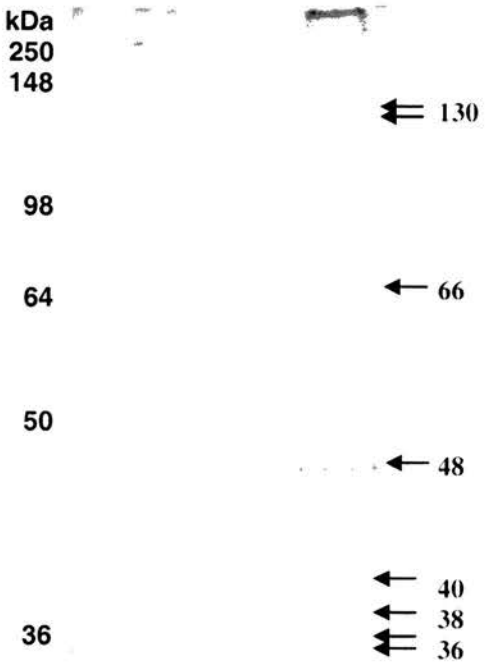
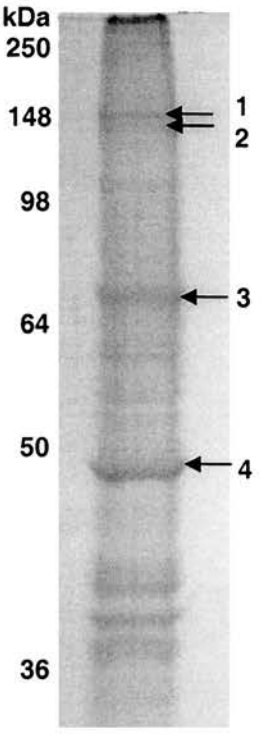


Figure 7.20: SDS-PAGE analysis of 0.08% CHAPS extracted AIHV-1 WC11 lysate. Thirty microlitre sample of CHAPS extracted lysate was run on a 12% bis-tris polyacrylamide gel and stained with SimplyBlue SafeStain. Approximate molecular weight of protein detected marked.



Band	Proteomic Hit
1	AlHV-1 orf25
2	AlHV-1 orf25
3	AlHV-1 orf21
4	AlHV-1 orf54

Figure 7.21: 0.08% CHAPS extracted AlHV-1 lysate analysed by Proteomic MALDI mass spectroscopy. Thirty microlitres of CHAPS extracted lysate was run on a 12% bis-tris polyacrylamide gel and stained with SimplyBlue Safestain. Bands indicated by the arrows were analysed by MALDI mass spectrometry.

7.4. Discussion

As the lysate-based ELISA described in chapter five proved effective, it raised the question of what antigens are being detected. This aim of this chapter was to identify potential MCFV antigens using several strategies such as affinity purification, chromatography and virus fractionation.

An initial identification of OvHV-2 antigens, recognised by clinical MCF antisera, was done by western blots, using an OvHV-2 infected cell. Although individual variations were seen in some cases, a similar pattern of reactivity was seen in the majority of samples, with strong reactive protein bands at 17, 28, 38, 40, 48, 62, 98, 140 and 188kDa. The major 140kDa component is assumed to be a nucleocapsid protein (Honess and Watson, 1977). Protein bands at 17, 28 and 62kDa were also recognised by MCF-negative sera. Therefore, as these bands were recognised by both MCF-positive and negative sera they can be eliminated for analysis as potential OvHV-2 antigens. A previous study analysing the reaction of cattle, sheep and wildebeest sera with AlHV-1 strain WC11 (Herring *et al.*, 1989), showed wildebeest sera from an animal infected with AlHV-1 reacted with six reactive polypeptides (32, 34, 35, 50, 77 and 140kDa), while bovine sera from an animal with AlHV-1 -induced MCF showed reactivity in a small proportion of samples tested, reacting to a small number of polypeptides (34, 35, 50 and 140kDa). This may suggest that cattle may only produce antibodies to a limited number of viral components as a result of either a low level of viral replication or an incomplete expression of the MCF virus DNA within the host (Herring *et al.*, 1989). It is also possible that in an MCF-infected animal, the immune system may either not have had time to make a complete response, due to a level of immunosuppression or the time of sampling. A further explanation for the difference in banding patterns observed between animals may be a result of variation in the major histocompatibility complex (MHC) restricted response shown by individual animals. The MHC is a main source of variation in immune responses and in cattle is termed the bovine lymphocyte antigen system, or *BoLA* (Lewin *et al.*, 1999). Differences in allelic forms of MHC may influence T-cell function and recognition of antigens.

Immunoprecipitation of virus-induced proteins was performed using infected and control rabbit polyclonal antibody in order to identify envelope and membrane proteins of the virus virion. Results suggested at least six glycoproteins as well as a non-glycosylated molecule possibly located in the viral envelope. Using monoclonal

antibodies, able to recognise virion envelope proteins, proteins of the gp115 complex, 48, 78, 105, 110 and 115kDa were shown to be antigenically related. Furthermore the 115kDa protein appears to be composed of two fragments, 48 and 78kDa proteins, linked by disulphide bonds. Ten viral-induced proteins, ranging from 48 to 145kDa, were shown of which two were assumed glycoproteins at 46 and 62kDa and a further two were cell proteins (45 and 200kDa) (Adams and Hutt-Fletcher, 1990).

Antiserum reacted with virions by immunofluorescence (Lahijani *et al.*, 1995) and a western blot detected three proteins at 15, 37-38kda and 55kDa. The 38 and 55kDa proteins were detected in both infected and control cell lysates. The 37kDa protein was only present in infected cells. A cDNA library, constructed using mRNA from AlHV-1 infected cells, was screened and identified a cDNA clone. The insert hybridised strongly with AlHV-1 DNA but did not hybridise with DNA from bovine herpesviruses 1, 2 or 4 and alcelaphine herpesvirus-2. This lack of cross-reactivity suggests that this sequence (ORF73) would be a good candidate for the development of an AlHV-1 specific diagnostic test, such as an ELISA or PCR. Similar work, by Coulter and Reid (2002) analysed the expression of viral antigens by western blotting, using polyclonal sheep serum. Using an OvHV-2 infected lymphoblastoid cell line, antigens at 60, 75 and a major antigen at 83kDa were identified.

In order to identify MCF-specific viral proteins, western blots were performed using infected cell lysates and sera from other bovine herpesviruses. While a previous study (Herring *et al.*, 1989) suggested BoHV-1 serum showed cross-reactivity with AlHV-1 polypeptides (25, 44, 57 and 65kDa), in this study all of the bovine herpesvirus antisera tested (BoHV-1, 2 and 4) showed the same pattern of reactivity as the IgG control. Bands were detected at 16 and 64kDa, suggestive of immunoglobulins. The AlHV-1 antiserum showed a distinct pattern of bands at 16, 25, 40, 55, 64, 98, 148 and 250kDa while the OvHV-2 antiserum detected only proteins bands detected by all bovine herpesviruses analysed. This may be due to the sensitivity of the test, or the optimisation of the serum samples using OvHV-2 lysate.

Analysis using an OvHV-2 lysate showed that both the experimental serum sample (AlHV-1) and the recovered clinical serum sample (OvHV-2) detected the same bands at 16, 32, 45, 48, 60, 62 and 64kDa. As this is latently infected cell lysate, there is a possibility that very little viral antigen is expressed. These bands are additionally detected by all of the bovine herpesvirus antisera tested. The lack of

unique bands reacting in the OvHV-2 whole cell lysate may be a result of the test sensitivity or lack of antigen expression by the cell line. The fact that the serum sample was from a clinical animal, allows the possibility of reactivity with other viruses and pathogens, and poses to the question of what MCF antigens are recognised by ELISA.

On analysis of the ELISA antigen by electrophoresis, six distinct protein bands at 45, 55, 64, 97, 100 and 188kDa were shown, while a western blot, using MCF-positive serum showed four bands expressed at 20, 50, 64 and 148kDa. Proteomic analysis of these bands, however, indicated a high level of host contamination which may have masked viral proteins.

Fractionation of the ELISA lysate was performed to isolate proteins of interest. A triple band at 97kDa and a band at 110kDa were identified as possible proteins specific to MCF. Proteomic analysis only identified a contaminant; bovine serum albumin. Fractionation of the OvHV-2 ELISA lysate was also performed and identified one strong protein band at 150kDa. Proteomic analysis identified an IgG associated protein, which suggests that contamination by host proteins may mask the presence AlHV-1 and OvHV-2 antigens. A possible explanation for the ineffective binding of the AlHV-1 lysate to the affinity or ion-exchange columns, may be the presence of Nonidet P-40 (Nonylphenylpolyethylene glycol), commonly used in extraction protocols to solubilize membranes. Nonidet P-40 is a non-ionic detergent and cannot be dialysed so that the concentration of detergent contained within the samples could be higher than recommended for chromatography.

Extraction using other detergents commonly used for cell lysis was performed on AlHV-1 WC11 maintained in serum-free medium. Protein bands could be discerned at 36, 38, 40, 48, 66, and a double band at 155kDa. Proteomic analysis identified ALHV-1 ORF21, ORF25 and ORF54. AlHV-1 ORF21 encodes a 569 amino acid protein, thymidine kinase homologous to HVS ORF21 (26%) and EBV BXLF1 (29%). AlHV-1 ORF25 encodes the 1370 amino acid major capsid protein. AlHV-1 ORF25 is homologous to HVS ORF25 (56%) and EBV BCLF1 (54%). AlHV-1 ORF54 encodes a 298 amino acid protein with homology to dUTPase (deoxyribouridine–triphosphate pyrophosphatase), an enzyme involved in viral replication and which is found in the virus particle (Chen *et al.*, 2002). AlHV-1 ORF54 is homologous to HVS ORF54 (36%) and EBV BLLF1 (27%).

Protein size (kDa)	**Length (aa)**	**ALHV-1 ORF**	*HVS* *% identity (similarity)*	*EBV* *% identity (similarity)*	Possible Function
20	136	ORF 52	51	-	Role in viral infection
48	298	ORF 54	36	27 (BLLF1)	Viral replication
66	569	ORF 21	26	29 (BXLF1)	Thymidine kinase
130	1370	ORF 25	56	54 (BCLF1)	Structural, Major capsid protein

Table 7.1: Summary of proteomic results

Using three approaches for the identification of potential MCFV antigens of diagnostic significance, a number of potential candidate recombinant antigens have been determined. Future studies would include the analysis of each of the AlHV-1 antigens: ORF21, ORF25, ORF52 and ORF54, identified in the crude viral extract with MCF-specific antibodies. Initial tests should be performed to define whether they are highly conserved within other bovine herpesviruses and therefore likely to be cross-reactive. A number of bands were additionally shown to be unique to MCF viruses by western blot using MCF antisera. Further analysis should be performed to identify these proteins.

The identification of these OvHV-2 genes as possible candidates offer encouraging possibilities for the development of a recombinant assay due to the evidence supporting their identification with positive MCF antiserum. Furthermore the identification of structural proteins, and in particular the major capsid protein, is particularly preferred as they are to be exported or expressed on the cell surface and therefore more likely to be exposed to the immune system.

Chapter Eight
General Discussion

8.0 Discussion

Accurate diagnostic tests for infectious diseases and viruses are crucial for the detection and control of most diseases (Peeling *et al.*, 2006). The diagnosis of a disease within an animal plays an important role in understanding disease transmission, prevalence of infection and the host immune response. In addition to the diagnosis of individual animals, tests are used for surveillance and monitoring of herd health status with an aim of establishing preventative measures and eradication programmes (Office International des Epizooties, 2006).

The primary objective of this study was to advance the current position in the diagnosis of OvHV-2. Current diagnostic methods include histopathological examination, indirect immunofluorescence test, virus neutralisation, conventional and real-time PCR assays and ELISAs. ELISAs are reliable, widespread, economical tests designed for the rapid throughput of samples for the specific detection of antibodies against an antigen such as MCFV. However, ELISA tests are often difficult to validate due to the signal amplification of specific and non-specific components. Methods and reagents may reduce assay specificity and increase test variability. Viral antigen derived from a whole-cell viral culture preparation may contain non-specific antigenic components. OvHV-2 lysate may contain non-specific antigens as a result of the method of preparation, including cellular proteins and serum proteins present in the culture medium. Development of a detection assay, using the recombinant expression of antigens, would therefore be beneficial for the specific detection of OvHV-2 infection.

Recombinant technology aiming at expression includes the identification, isolation and expression of genes in non-host organisms such as bacteria, yeast, insect cells and eukaryotic cells. Large quantities of specific gene product may be obtained as recombinant proteins. Ideal candidate antigens are natural proteins which are major stimulants of cellular and humoral immunity against the disease, such as structural epitopes, which may be continuously produced in both infected animals and cell culture (Office International des Epizooties, 2006). Prerequisites of recombinant antigens for use in immunoassays include the reproducibility of the antigen and the purity. The production of large amounts of antigen is favoured for ensuring reproducible results within a diagnostic assay. As many proteins are present in recombinant expression systems, purification is required to remove potential non-specific antigenic targets (Schmitt and Papisch, 2002; Schmitt, 2003).

The determination of the DNA sequence for the OvHV-2 genome (Hart *et al.*, 2007) has confirmed the identification of twelve genes unique to MCF viruses and three genes unique to OvHV-2 (Table 1.2, Figure 1.10). The use of such unique genes for a diagnostic assay would be advantageous as it would reduce potential cross-reactivity with other herpesviruses. The OvHV-2 genes selected for analysis in this study were divided into three categories. Genes were firstly selected on the basis of use in diagnostic approaches in related herpesvirus systems. Secondly, of the MCF specific genes, potential antigens were selected if they were expressed on the surface of the virus, as polypeptides likely to be exported or expressed on the cell surface may be exposed to the immune system. Finally genes which were unique to the OvHV-2 genome were selected as they are potentially less likely to cross-react with other herpesviruses. Knowing the sequence of the OvHV-2 genome enabled PCR primers to be designed to amplify candidate genes or gene fragments and create in-frame fusions when cloned into the expression vectors. Bacterial systems are simple, stable, continuous systems for the production of large quantities of protein. For the expression of candidate genes, bacterial expression systems were selected that could add an epitope tag, HisTag, to aid purification. Bacterial systems showed expression of glycoprotein Ov8 and capsid protein ORF65 both of which appeared to be recognised by anti-MCF sera. Purification by affinity chromatography using an epitope tag was ineffective in all cases attempted. In the absence of a clean purification method, a diagnostic test would not be deemed reliable due to the possibility of false-positive results generated from impurities. The candidate genes were subsequently not analysed by ELISA due to the lack of protein expressed and purity.

Disadvantages of the bacterial systems include the formation of insoluble aggregates and the need for purification. Purification of recombinant antigens is essentially due to the presence of antibodies against *E. coli* proteins in most livestock. Furthermore the fusion partner may be antigenic. In the case of the thioredoxin expression system, the thioredoxin protein may cross-react with the fusion proteins expressed. The alternative approach of the *in vitro*, novel rapid translation system resolved solubility issues regarding the expression of some OvHV-2 proteins, but, an improved purification method is required to separate the target protein from potential non-specific cross-reacting proteins in the *E. coli* extract. It can be concluded from this initial study, that in order to develop a diagnostic assay based on recombinant proteins, further work on protein expression

is required and specifically, improved purification of target proteins is needed before work using MCF-specific serum may be attempted. Future studies on the expression of recombinant OvHV-2 antigens should include the investigation of different expression systems. Unlike prokaryotic hosts, the eukaryotic cellular organisation of yeast expression systems allow for post-translational folding and modification of the protein to mammalian proteins with native epitopes (Schmitt, 2003). Further advantages of using a yeast system include rapid growth in culture and the relative simplicity of genetic manipulation (Buckholz and Gleeson, 1991). Insect cells/baculovirus expression systems are also commonly used for the expression of proteins. Baculovirus expression systems have been used in the characterisation and assembly of the herpes simplex virus capsid (Newcomb *et al.*, 1996), and in the expression of KSHV latent and lytic genes, capsid protein ORF65 and glycoprotein K8.1 (Zhu, *et al.*, 1999). Mammalian cell systems offer ideal expression conditions for mammalian antigens. An example of the effective expression of antigens using mammalian systems has been shown for Epstein-Barr virus, with the successful expression of the glycoprotein gp350/220 in rodent and primate cells (Whang *et al.*, 1987). Subsequent studies should therefore include the analysis of the glycoprotein Ov8 and capsid protein ORF65 in eukaryotic expression systems, with an aim to resolving solubility and purification issues, as these antigens show potential for recombinant assay development.

Recombinant antigens and synthetic peptides (Davis *et al.*, 1997; Pau *et al.*, 1998) have been used in ELISA development for the detection of antibodies against KSHV. Immunoreactive lytic-cycle proteins were considered as potential candidates for serological analysis (Simpson *et al.*, 1996). ORF52 and ORF65 segments were analysed in bacterial vectors (pEQ 42 HisTag), although only the capsid protein, ORF65, showed reactivity to anti-KSHV sera. ORF52 and ORF65 are homologues of EBV genes BLRF3 and BFRF3 respectively. The carboxyl terminal of ORF65 (ORF65.2) was used as an ELISA antigen and showed over 80% of KS sera were reactive. On comparison with immunofluorescence, a percentage of KSHV-positive sera were missed by both assays. Antibodies to the capsid protein were not detected in all cases of KSHV and suggest that for a more sensitive method, a combination of antigens would be required for an accurate diagnosis (Simpson *et al.*, 1996).

Assays were additionally developed using synthetic peptides of unique sequence from the capsid proteins ORF26 and ORF65. These peptide-based assays showed similar findings to those of the recombinant-based ELISA. The general lack of

sensitivity may be a result of the restricted antigenic epitopes in the peptide assay, where patients may not have mounted a specific antibody response. The poor general sensitivity of the ELISA may be due to the candidate peptides selected. ORF26 and ORF65, like their EBV homologues, are only produced during lytic infection and may not be appropriate to detect long-standing latent infections. Using discrete peptides and single recombinant antigens, assays may miss antibodies to immunogenic viral proteins present in the virus. Also, individuals may differ in the main focus of their antibody response. A highly sensitive assay for the detection of KSHV infection may, therefore, require a cocktail of peptides or proteins derived from both lytic and latent infections (Davis *et al.*, 1997; Pau *et al.*, 1998). Latent antigen (ORF73) and lytic antigens (ORF65, K81A and K8.1B) were expressed in both bacterial and baculovirus-insect cell expression systems and purified as GST fusion proteins. All proteins reacted with anti-KSHV sera by western blotting. Increased sensitivity was shown for the lytic proteins. This may be explained by a higher number of immunogenic proteins being produced during lytic infection. Antigens recognised by sera were shown to vary. This difference in the detection of antibodies during infection may be due to a number of factors, such as low antibody titres and differences in ability to recognise proteins in individual patients. Low antibody titres may be a result of the pathogenesis of infection, in which only limited replication occurs. A comparison of western blotting and immunofluorescence indicate this group of latent and lytic-associated proteins may be employed to detect serological responses to KSHV infection (Zhu *et al.*, 1999).

Despite being a rapid and specific approach for the development of a diagnostic assay, recombinant expression antigen assays have their disadvantages. Problems with protein expression can occur due to instability, insolubility or toxicity of the recombinant protein in an expression vector. Furthermore, unless several recombinant proteins are used for diagnosis, the assay focuses on a single epitope which may or may not be recognised by an individual's immune response. A general lack of sensitivity may be a result of the lack of sufficient time required to mount an immune response due to the rapid clinical progression of infection. It is therefore possible that individuals may lack humoral immune responses to the viral antigen targeted in the assay (Li *et al.*, 1996). Analysis of OvHV-2 lysate by western blot, using a range of MCF antiserum samples identified a number of antigenic proteins. The majority of sera were shown to recognise a number of distinct proteins, with strong reactive bands at 17, 28, 38, 40, 48, 62, 98, 140 and 188kDa. Variation,

however, was seen between individual serum samples. Possible explanations for these variations include a variation in the duration of infection, variation in MHC responses shown by individual animals or a difference in the level of viral replication. This difference in antigens recognised by serum samples indicates potential difficulties in the specific diagnosis of MCF infection. Therefore, a large pool of animals would have to be tested before an antigen could be assumed to reliably indicate disease.

There are, therefore, advantages to using a crude antigen in diagnostic tests. These include the low cost of production, increased antigen yield and the detection of a wider range of antibody specificities. An ELISA was developed for the detection of antibodies against KSHV using whole-cell viral lysate, prepared from the KS-1 cell line. Using this crude virus lysate, it was assumed that viral structural and non-structural proteins are present, with both lytic and latent proteins represented. This assay was specific and detected antibodies present in the sera before infection developed (Chatlynne *et al.*, 1998) and showed good correlation with immunofluorescence assays (Topino *et al.*, 2001). Further studies using whole-antigen have also proved effective (Baeten *et al.*, 2002; Cholan *et al.*, 2004) in studying the prevalence of KSHV in Kenya.

In the absence of sufficient recombinant antigen expression, trial ELISA experiments were done using whole-cell viral culture preparations from cultured AlHV-1 and OvHV-2 infected cells. The crude antigen ELISA relies on a polyclonal response and may consequently be more robust where the infected animal has limited or low antibody production. Evaluation of extracts showed that only the AlHV-1 antigen could be used to differentiate MCF-positive and negative sera and showed good concordance to other MCF diagnostic tests (Fraser *et al.*, 2006). The OvHV-2 lysate from an infected cell culture did not show any ELISA signal and so did not appear to express antigens suitable for the detection of MCF virus specific serum antibodies. OvHV-2 infects cells productively before establishing latency (Thonur *et al.*, 2006). A difficulty with OvHV-2 is the lack of a productive culture system for propagating virus, so that analysis of the virus replication cycle is limited (Rosbottom *et al.*, 2002). OvHV-2 can be cultured in an LGL cell-line infected lymphoid cells derived from MCF affected animals. On analysis by ELISA, OvHV-2 antigens from the LGL cell line proved ineffective for the analysis of MCFV infection. Possible reasons for this inability to discriminate between positive and negative samples may have been the result of a lack of an adequate negative control

antigen. In the case of the AlHV-1 antigen, which is prepared in bovine turbinate cells, mock infected cells can be used as a suitable control. As a control is required for an ELISA experiment, with no OvHV-2 negative LGL cell line available, a suitable control had to be established. Concanavalin A (Con A)-treatment of peripheral blood lymphocytes has been shown to stimulate proliferative responses. The use of ConA stimulated blast cells from a known MCF-negative animal were therefore used as a control, representing activated bovine cells without infection. An additional control should have been the inclusion of lymphocytes not stimulated by ConA, so the level of background from the stimulated cells could be analysed. On analysis with an MCF-positive serum, the signal for the ConA stimulated cells was equal to the OvHV-2 infected cells. This suggests a lack of antigens expressed within the OvHV-2 lysate. On analysis of the OvHV-2 lysate by gel filtration, there was a significant difference in the antigens that were recognised by an MCF-positive antiserum compared to those recognised in the AlHV-1 lysate.

OvHV-2 infected bovine LGLs have been shown to predominantly contain circular genomes with linear genome scarcely detectable (Rosbottom *et al.*, 2002; Thonur *et al.*, 2005). In contrast rabbit LGLs contained mainly linear genomes. This suggests that the OvHV-2 infected cells used in these ELISA tests contained predominantly latently infected cells (Rosbottom *et al.*, 2002) and may explain the lack of suitable antigens within the crude lysate.

Future studies should include the continued validation of the established AlHV-1 based ELISA assay. The development and validation of a diagnostic assay is essentially comprised of five phases; (1) the feasibility of the method for a particular use, (2) optimisation and standardisation of reagents and techniques, (3) determination of assay's performance characteristics (4) continuous monitoring of assay performance and (5) maintenance of validation criteria during routine usage (Figure 8.1). Initial feasibility studies have been carried out to establish a selected protocol and assess whether reagents possess the ability to discriminate between ranges of antibody concentrations, in the presence of minimal background interference. By carrying out such studies, an insight into the estimated repeatability, test sensitivity and specificity may be gained (Office International des Epizooties, 2006). In order to calculate the assay sensitivity and specificity, it is recommended that at least 300 reference samples from known-infected animals and 1000 samples from known uninfected animals should be tested to give an accurate test sensitivity and specificity (Office International des Epizooties, 2006). In the development of the

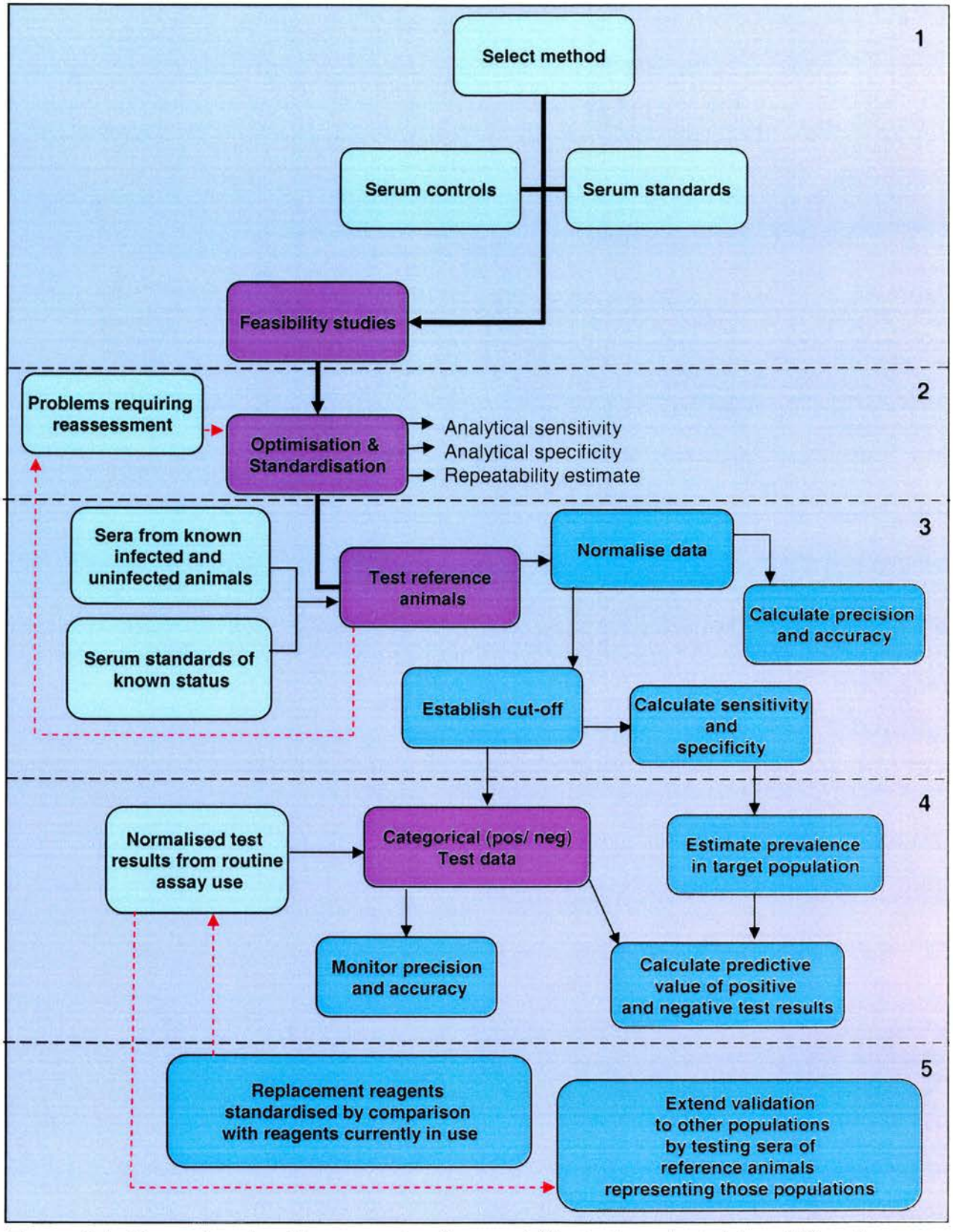


Figure 8.1: Stages involved in assay development (Illustration adapted from Office des Epizootes, 2006).

virus-based ELISA, approximately 100 known-infected animals and 350 samples from known uninfected animals have been tested. Therefore only an estimated sensitivity and specificity can be confirmed. An important stage in determining test sensitivity and specificity is the estimated prevalence in a target population, in order to gain predictive positive and negative test results. In the absence of test sensitivity and specificity, predictions regarding the infection status can not be drawn. Characterisation of the assay should include the continued sampling of known infected and uninfected animals and may include the sampling of reservoir hosts wildebeest and sheep to further assess test sensitivity. The preliminary analysis of cattle experimentally infected with AlHV-1 and natural MCF outbreaks suggest a high level of sensitivity. A further objective should be the determination of prevalence of antibodies against MCFV in domestic and wild ruminants, using the developed ELISA (Fraser *et al.*, 2006). Once an assay has been validated, it requires constant monitoring and maintenance. This may be performed by internal controls to assess the tests repeatability and accuracy. External controls may also be tested. Reference laboratories distribute panels of samples to several laboratories using the same test. If the test results are found within acceptable limits, with evidence of accuracy and reproducibility, the laboratory receives certification as an official laboratory for that assay. With the constant need for reagents, it is essential to test replacement reagents to establish new test conditions. If the assay is moved to a new geographical location, re-evaluation must be performed. This may result in a difference in population, under local conditions.

Validation of a diagnostic serological test routinely involves the comparison of the new test with a method or combination of methods, routinely used. The routine method may therefore be termed as a gold standard. The term "gold standard" should only be limited to methods that clearly classify animals as infected or uninfected (Office International des Epizooties, 2006). In the absence of a reliable gold standard, the validation of a new test may involve the comparison of multiple test methods. In this case, all tests are assessed equally. A reliable diagnosis of MCF may only be guaranteed by histopathology. Therefore, in the absence of histopathological examination, a comparison of diagnostic tests may indicate a general trend in the ability of a correct diagnosis. Four diagnostic tests for MCF were tested and showed good agreement. Of particular interest in this preliminary validation study, a group of samples were shown to be DNA positive but serology negative. These samples are of particular interest, suggesting the presence of virus within an individual that has not

developed an immune response. A second group of interest include DNA negative, serology positive samples. These samples may indicate cases of recovered or latent viral infection. These samples may indicate the possibility of false-negative samples in both DNA tests and serological tests and, therefore, highlight the need for further validation.

In parallel with validation studies, future studies should include the continued analysis of the antigenic components contained within the crude AlHV-1 lysate, with the aim of identifying possible candidates for expression as recombinant antigens. In initial experiments for the identification of antigenic components of the ELISA, western blotting with anti-MCF antiserum was used to guide fractionation of the ELISA antigen by gel filtration, ion exchange and affinity chromatography. Clear fractionation of antigenic bands in the lysate was detected but subsequent proteomic analysis identified host proteins. One virus antigen was identified as ORF52. AlHV-1 ORF52 is a homologue to KSHV ORF52, EBV BLRF3 and the HSV-1 outer tegument protein VP22 (UL49). It is assumed that the majority of tegument is added to the HSV-1 capsid in the cytoplasm. Capsids are assembled in the nucleus before they cross the inner and outer nuclear membrane. At this stage of assembly, the inner tegument proteins are added to the capsid, while outer tegument proteins interact with the cytoplasmic tail of viral glycoproteins. The outer tegument and membrane-anchored glycoproteins then form the final viral particle. HSV ORF52 is therefore thought to play a structural role in HSV assembly (Vittone *et al.*, 2005).

In contrast, sequential detergent extraction of AlHV-1 strain WC11 revealed several antigenic bands, ORF21, ORF25 and ORF54. This finding may suggest a means towards the identification of the antigenic components of the ELISA and possible candidates for recombinant studies. It may also suggest a method for fractionating the lysate for an improved ELISA method. AlHV-1 ORF21 is homologous to HSV ORF21 (HSV UL23) which encodes viral thymidine kinase. AlHV-1 ORF54 is homologous to HSV UL50 which encodes dUTPase, located in the viral particle. AlHV-1 ORF25 is a major virus capsid protein, with homology to HSV ORF25 (UL19). The major capsid protein, VP5, coded by the UL19 gene, is a predominant structural subunit of hexons and pentons.

Studies of KSHV diagnostic assays demonstrated that a recombinant based assay might not confidently identify all cases of infection if the assay was solely based on a lytic-phase protein. Zhu *et al.*, (1999) determined that expression of a complex of proteins, comprised of both latent and lytic-phase proteins, may be

beneficial for the diagnosis of infection. The four genes identified through proteomic analysis of the whole-cell viral lysate were shown to be proteins expressed during lytic replication, ORF21, ORF25, ORF52 and ORF54. The identification of only lytic proteins may simply be a result of the lysate being prepared from a lytic culture. From the KSHV literature it seems likely that an assay based on only lytic proteins may not accurately diagnose all cases of MCF infection, although lytic antigens are seen by MCF sera. KSHV antiserum was shown to react with the latency-associated nuclear antigen, KSHV ORF73, (Zhu *et al.*, 1999). The inclusion of a latent protein such as ORF73 in a recombinant antigen based assay, or added to the crude lysate assay, may therefore be beneficial. During the construction of a cDNA library generated from lymphoblastoid cell lines, a clone containing a partial ORF with similarity to AlHV-1 ORF73 was identified (Lahijani *et al.*, 1995; Zhu *et al.*, 1999; Coulter and Reid, 2002). On analysing the reactivity of the OvHV-2 ORF73 C-terminal fragment, against sera from OvHV-2 infected sera, the fusion protein was recognised by both MCF positive and negative bovine sera. This indicated the ORF73 antigen may not be suitable for use in the diagnosis of OvHV-2 infection.

Future studies would include the analysis of each of the four AlHV-1 antigens identified in the crude viral extract with MCF-specific antibodies. A further consideration for the suitability of these genes in a diagnostic test is to define whether they are highly conserved within other bovine herpesviruses and so likely to be cross-reactive. ORF25 and ORF54 are conserved within all herpesviruses while ORF21, ORF52 and ORF73 are conserved within gammaherpesviruses. These highly conserved genes may therefore not be suitable for the detection of MCF-specific antibodies. AlHV-1 ORF25 has recently been expressed in mammalian cells, but was not detected by MCF sera on western blot suggesting that ORF25 is not a major antigen (Russell and Dasari, unpublished finding). In contrast, glycoprotein B expressed in the same system was shown to be antigenic by MCF-positive sera and therefore may be a candidate recombinant antigen. Glycoprotein B (gB) of AlHV-1, encoded by ORF8, is one of the most conserved herpesvirus glycoproteins (Dunowska *et al.*, 2001) and plays a role in viral entry. Expression of a band at approximately 105kDa was detected using IgG purified from AlHV-1-infected bovine sera. The MAb 15A used in the commercially available CI-ELISA binds an epitope located on a glycoprotein complex which, on reducing polyacrylamide gels, resolves into five bands at 45, 78, 105, 110 and 115kDa. Proteomic analysis of AlHV-1 virions (Dry *et al.*, unpublished observation) has shown gB is present in the

mature virion as 2 furin-cleaved polypeptide complexes of 50 and 80kDa, and suggests MAb 15-A recognises gB. MAb 15A also recognises the recombinant gB expressed in mammalian cells. Thus gp115, a highly conserved and immunogenic complex (Adams and Hutt-Fletcher, 1990) seems to include gB. Further work should therefore include the analysis of glycoprotein B, for its potential as a diagnostic antigen for the detection of antibodies against MCFV.

In conclusion, while this study has not described the development of a diagnostic test specific for ovine herpesvirus-2, it has identified a number of candidates that may provide a starting point for future experiments. The development of a working MCF ELISA based on a crude AlHV-1 infected-cell lysate has facilitated alternative methods for the identification of antigenic proteins. Additionally, a number of reagents have been generated, including recombinant antigens and crude MCF lysates, which could prove useful in other investigations.

This work will offer new contributions to scientific knowledge within the field of MCF, as not only has the crude antigen ELISA (WC11-ELISA) the potential to offer a rapid alternative to the diagnosis of disease, the discovery of potential recombinant antigens offers the development of a recombinant based assay which may prove more sensitive in its diagnosis. The ELISA will firstly be assessed and validated for its reproducibility in a commercial market. On validation, the assay should benefit studies regarding the continued immune status of animals, studies for screening animals before entering an MCF free herd and for the addition to exotic collections. ELISA validation is currently ongoing.

Analysis of several candidate antigens performed during this thesis may prove advantageous to others studying this field, whereby these candidates may be discounted. The negative findings of this study were presented at PhD meetings, and hopefully may offer information regarding the solubility issues observed when using the described expression methods. Furthermore, the work performed on the development of the crude antigen ELISA was presented at veterinary meetings and a herpesvirus conference workshop.

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ULR 1: Primer3 server: frodo.wi.mit.edu/

URL 2: SignalP 3.0 Server: www.cbs.dtu.dk/services/SignalP/

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Appendix 1

RM Medium

6g Na_2HPO_4 (anhydrous)
3g KH_2PO_4
0.5g NaCl
1g NH_4Cl
20g Casamino acids
0.095g $MgCl_2$
Add deionized H_2O to a final volume of 1 liter
Autoclave
Add 2.5ml sterile 20% dextrose and 1 ml of 100 mg/ml filter-sterilized ampicillin
Pour into petri dishes (~25 ml/100-mm plate)

SOC Medium (per 100 ml)

2 ml of filter-sterilized 20% (w/v) glucose or 1 ml of filter-sterilized
2 M glucose
SOB medium (autoclaved) to a final volume of 100 ml

SOB Medium (per Liter)

20.0g of tryptone
5.0g of yeast extract
0.5g of NaCl
Add deionized H_2O to a final volume of 1 liter
Autoclave
Add 10 ml of filter-sterilized 1 M MgCl2 and 10 ml of filter-sterilized

LB Agar (per Liter)

10g of NaCl
10g of tryptone
5g of yeast extract
20g of agar
Add deionized H_2O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave

LB–Ampicillin Agar (per Liter)

1 liter of LB agar, autoclaved
Cool to 55°C
Add 10 ml of 10 mg/ml filter-sterilized ampicillin
Pour into petri dishes (~25 ml/100-mm plate)